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STRUCTURAL ANALYSIS OF CALRETICULIN

BY

SHAIRAZ BAKSH ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Sciences (Pediatrics)

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ABSTRACT

Calreticulin is a major Ca^{2+} sequestering protein of pancreatic ER. It was purified from canine pancreas using a selective ammonium sulfate precipitation procedure, followed by cation exchange chromatography. Recombinant calreticulin and calreticulin domains (the N-domain, P-domain, and the C-domain) were expressed in a Glutathione S-transferase (GST) prokaryotic expression system. Canine pancreatic calreticulin was not significantly glycosylated and was not phosphorylated by protein kinase A, protein kinase C, and casein kinase II. It was found to contain a single intramolecular disulfide bond.

Using equilibrium dialysis, calreticulin was determined to contain two distinct Ca^{2+} binding sites: a single low capacity, high affinity site and high capacity, low affinity sites. Using recombinant calreticulin domains, the P-domain of calreticulin contained the low capacity, high affinity Ca^{2+} binding site and the C-domain of calreticulin contained the high capacity, low affinity Ca^{2+} binding sites. Furthermore, it was determined that the three repeat sequence A segments (each containing the sequence PXXIXDPDAXKPEDWDE) within calreticulin's P-domain may be required for high affinity Ca^{2+} binding to the protein. The N-domain was shown not to bind Ca^{2+}.

Using a Zn^{2+} specific dye, calreticulin was shown to contain two distinct binding sites for Zn^{2+}: low capacity, high affinity sites and high capacity, intermediate affinity sites. Using recombinant calreticulin domains, we observed that the N-domain of calreticulin contained the low capacity, high affinity Zn^{2+} binding sites and the C-domain of

calreticulin contained the high capacity, intermediate affinity Zn^{2+} binding sites. The P-domain was shown not to bind Zn^{2+}. Ca^{2+} did not affect calreticulin's Zn^{2+} binding ability indicating that calreticulin has distinct Zn^{2+} and Ca^{2+} binding sites.

While investigating calreticulin's Zn^{2+} binding site, protein disulfide isomerase (PDI) was observed to copurify with canine pancreatic calreticulin. Both calreticulin and PDI were found to modulate each other's function. Using GST-affinity chromatography, it was shown that two contact points exist within calreticulin for PDI - the N-domain and the P-domain. Calreticulin is thus a highly domain structured protein with each domain performing distinct functions.

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LIST OF ABBREVIATIONS AND SYMBOLS

ATP	adenosine triphosphate
BiP	immunoglobin heavy-chain binding protein
bp	base pairs
Ca^{2+}	calcium
cDNA	complementary deoxyribonucleic acid
CNBr	cyanogen bromide
CRT	calreticulin
DEAE	diethylaminoethyl
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
dNTP	deoxynucleoside triphosphate
DTNB	5, 5′-dithiobis-(2-nitrobenzoic acid)
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
EGTA	ethylene glycol bis(β-aminoethyl ether) N, N, N', N'-tetraacetic acid
ER	endoplasmic reticulum
FPLC	fast protein liquid chromatography
GlcNAc	N-acetyl-glucosamine

GST	Glutathione S-transferase
HA	high affinity
HEPES	N-2-hydroxyethypiperazine-N'-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
IgG	immunoglobulin G
$InsP_3$	inositol 1,4,5-trisphosphate
IPTG	isopropyl β-D-thiogalactopyranoside
JSTX	joro spider toxin
kb	kilobase
K_d	ligand concentration that gives high maximal binding
kDa	kilodalton
LB	Luria-Bertani
Mg^{2+}	magnesium
MCS	multiple cloning site
MOPS	4-morpholinepropanesulfonic acid
M_r	molecular weight
mRNA	messenger ribonucleic acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDI	protein disulfide isomerase
PMSF	phenylmethylsulfonyl difluoride
PVDF	polyvinylidene difluride
SACH	salicylcarbohydrazone
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SR	sarcoplasmic reticulum
TBS	tris-buffered saline

TE	Tris-EDTA
TPEN	tetrakis(2-pyridylmethyl)ethylenediamine
UV	ultraviolet
Zn^{2+}	zinc

DNA abbreviations

A	adenine
C	cytosine
G	guanine
T	thymine
Y	uracil

Protein abbreviations

A	alanine
C	cysteine
D	aspartate
E	glutamate
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine

N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
x	any amino acid
Y	tyrosine

CHAPTER ONE

Introduction

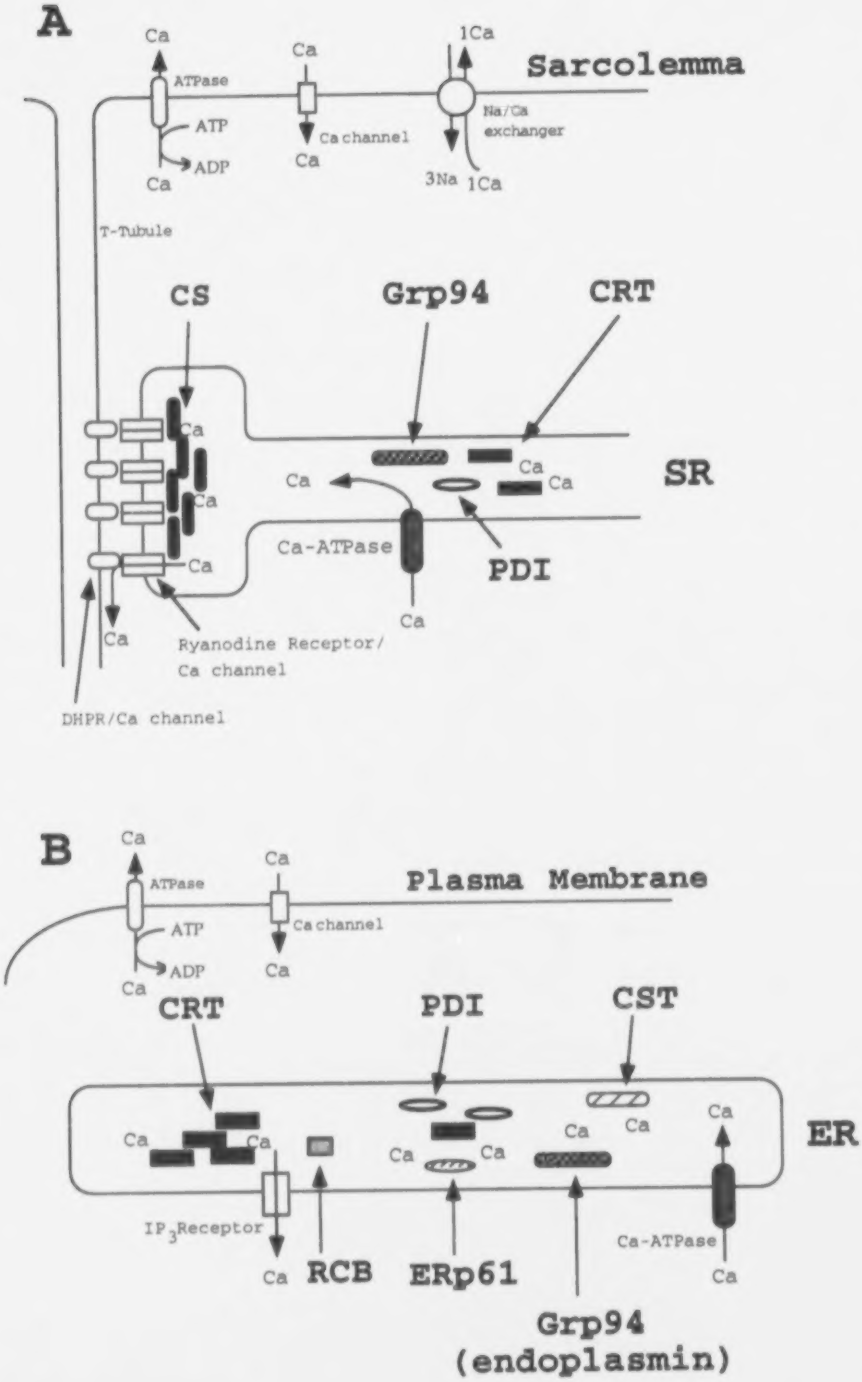
Introduction

Regulation and control of Ca^{2+} homeostasis is of paramount importance for all eukaryotic cells. Ca^{2+} ions play a vital role in a wide variety of cellular processes (Carafoli, 1987). These processes are controlled by changes in the concentration of intracellular cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) which is maintained at a level that is 10 000 fold lower than the extracellular milieu (Pietrobon *et al.*, 1990). Changes in $[Ca]_i$ not only controls specialized cellular functions such as excitability, muscle contraction, or exocytosis but also plays a key role in regulating universal cellular activities such as many aspects of cellular metabolism and gene expression. Therefore, Ca^{2+} is an important intracellular signaling molecule. In most cells, elevations in $[Ca]_i$ arise from Ca^{2+} entry via the Ca^{2+} channel in the surface membrane, or Ca^{2+} discharge from internal stores, or both (Inesi, 1985). One of the important mechanisms for maintaining $[Ca]_i$ in the sub-micromolar range involves the plasma membrane Ca^{2+}-ATPase systems, which pumps two Ca^{2+} ions actively out of the cytoplasm at the expense of an ATP molecule (Carafoli, 1987) (Fig. 1-1A). The plasma membrane Na^+/Ca^{2+} exchanger of excitable cells is also known to function together with the Ca^{2+}-ATPase during membrane repolarization to cause Ca^{2+}efflux - pumping one Ca^{2+} ion out for three Na^+ ions in (Pietrobon *et al.*, 1990) (Fig. 1-1A). During membrane depolarization, the Na^+/Ca^{2+} exchanger works together with the Ca^{2+} channel to cause Ca^{2+} influx - pumping one Ca^{2+} ion in for three Na^+ ions out (Pietrobon *et al.*, 1990). Therefore, two main types of Ca^{2+}-extruding systems are known - one unidirectional (the Ca^{2+}-ATPase) and the other bi-directional (the Na^+/Ca^{2+} exchanger).

Figure 1-1: Ca^{2+} storage proteins in SR and ER membranes of (A) muscle and (B) non muscle cells

CS, calsequestrin; CRT, calreticulin; PDI, protein disulfide isomerase; CST, ERcalcistorin/calstorin; Grp94, glucose regulated protein or endoplasmin; ERp61, ER protein 61; RCB, reticulocalbin; DHPR, dihydropyridine receptor

Adapted from Milner *et al.* (1992).


A
Ca
ATPase
ATP
ADP
Ca
Ca
Ca channel
Ca
1Ca
Na/Ca exchanger
3Na
1Ca
Sarcolemma
T-Tubule
CS
Grp94
CRT
Ca
Ca
Ca
Ca
Ca
Ca
SR
Ca-ATPase
PDI
Ca
Ca
Ryanodine Receptor/ Ca channel
DHPR/Ca channel
B
Ca
ATPase
ATP
ADP
Ca
Ca
Ca channel
Ca
Plasma Membrane
CRT
PDI
CST
Ca
Ca
Ca
Ca
Ca
Ca
Ca
ER
IP$_3$Receptor
Ca
RCB
ERp61
Grp94 (endoplasmin)
Ca-ATPase
Ca

It is well established that intracellular membrane systems also play an important role in sequestering and storing significant amounts of Ca^{2+}. One of the best characterized intracellular system operating under normal physiological conditions is the sarcoplasmic reticulum (SR) in the muscle (MacLennan Holland, 1975; Lytton and Nigam, 1992). The SR is generally regarded as a specialized form of the endoplasmic reticulum (ER), and functions to remove Ca^{2+} from muscle cytoplasm (via the membrane Ca^{2+}-ATPase) during relaxation, and release Ca^{2+} to the muscle cytoplasm during contraction (via the Ca^{2+}-channel) (see Fig. 1-1A). It is becoming apparent that there is analogy between the proteins responsible for Ca^{2+} uptake across the SR membrane systems of cardiac and striated muscle and the ER membrane systems of smooth muscle and non-muscle cells. For example, Ca^{2+} uptake into pancreatic ER membranes is known to be mediated by a Ca^{2+}-ATPase that resembles Ca^{2+}-ATPase of SR in cardiac muscle (Burke *et al.*, 1989); and within non-muscle cells, two isoforms of the SR Ca^{2+}-ATPase exist (Burke *et al.*, 1989). These observations prompted the redefinition of this class of enzymes as the "sarcoplasmic or endoplasmic reticulum Ca^{2+}-ATPase" - the SERCA Ca^{2+} pumps (Burke *et al.*, 1989; Lytton *et al.*, 1988). The functional significance of having these two different non-muscle enzymes (SERCA2b and SERCA3) is currently unknown. Localization of SERCA2b is ubiquitous and is expressed more abundantly than SERCA3 whose expression is only predominantly higher in the large intestine and cerebellum (Lytton and Nigam, 1992). Nevertheless, both isoforms appear to be required for Ca^{2+} uptake in their respective environments.

In skeletal and cardiac muscle Ca^{2+} uptake into the SR is believed to be stored in the lumen of these membranes bound to calsequestrin, a low affinity, high capacity Ca^{2+} binding protein (MacLennan *et al.*, 1983). This high capacity Ca^{2+} binding protein is specifically localized to the lumen of the junctional SR (Fig. 1-1A) and is believed to closely juxtaposed to the ryanodine receptor/Ca^{2+} channel (Fig. 1-1A). This close association thus provides rapid access to stored Ca^{2+} by the ryanodine receptor/Ca^{2+} channel. Calsequestrin is predominantly confined to cardiac and striated muscle cells (Milner *et al.*, 1991). The ryanodine receptor/Ca^{2+} channel, however, is found at low levels in the brain and smooth muscle cells (Otsu *et al.*, 1990) and in many non-muscle and non-neuronal tissues (Tsien *et al.*, 1990). Several lines of data thus suggest that the ryanodine receptor/Ca^{2+} channel may be more ubiquitous than originally believed. Figure 1-1A shows that in addition to calsequestrin, other Ca^{2+} binding proteins may contribute to Ca^{2+} storage within the SR of muscle cells (Milner *et al.*, 1992).

Within non-muscle cells the predominant mechanism for release of stored Ca^{2+} is triggered by a diffusible messenger, inositol 1, 4, 5-trisphosphate ($InsP_3$). $InsP_3$ binds to an integral ER membrane protein, the $InsP_3$ receptor, thereby triggering the mechanism of $InsP_3$-induced Ca^{2+} release (Clapman, 1995) (Fig. 1-1B). The $InsP_3$ inducible Ca^{2+} release mechanism only accounts for release of 30 - 50 % of the total sequestered Ca^{2+}, while the remaining pools of Ca^{2+} may be released by a variety of agonists including GTP, Ca^{2+} itself, caffeine, and inositol tetrakisphosphate ($InsP_4$) (Tsien and Tsien, 1990; Irvine, 1992; Taylor and Marshall, 1992). The $InsP_3$ receptor exhibits a high degree of structural homology to the ryanodine receptor/Ca^{2+} channel present in SR membranes of skeletal and cardiac muscle (Mignery *et al.*, 1989). Both channels function as a quaternary homotetrameric molecule and share limited amino acid sequence similarity within their carboxyl-terminal region (Tsien and Tsien, 1990). Internal stores within the ER of non-muscle cells are believed to be intimately connected to the $InsP_3$ receptor, enabling rapid access to stored Ca^{2+} that is required for physiological functions (Taylor and Marshall, 1992; and Lytton and Nigam, 1992). Research over the last ten years have yielded a substantial amount of information concerning the identity and location of these internal stores and how they respond to external agonists such as $InsP_3$, GTP, and caffeine.

The identity of some of the protein components of these internal Ca^{2+} stores in the lumen of the ER of non-muscle cells are known. The total Ca^{2+} concentration within the ER can approach the millimolar range, but the cell does not allow for this Ca^{2+} to remain in free form. Most of the lumenal Ca^{2+} is believed to be bound to lumenal Ca^{2+} binding proteins. Therefore, the free Ca^{2+} concentration in the lumen of the ER (and SR) may be in the sub-micromolar range. Koch (1987) proposed to name the proteins responsible for buffering the Ca^{2+} in the lumen of the ER by a collective name, the reticuloplasmins, to reflect their intracellular localization. A reticuloplasm-rich extract isolated from a murine plasmacytoma cell line contained large amounts of ER with five major Ca^{2+} binding proteins of apparent molecular weights of 100,000, 75 000, 60 000, 58 000 and 55 000 (Macer and Koch, 1988 and Table 1-1). Components of the reticuloplasmins were later identified

Table 1-1: Ca^{2+} binding proteins in the lumen of the ER

References:

1. Mazzarella and Green, 1987; **2.** Koch *et al*, 1985; **3.** Lee *et al*, 1984; **4.** Koch *et al*, 1985 and 1986; **5.** Lee *et al*, 1981; **6.** Haas and Wabl, 1983; **7.** Munro and Pelham, 1986; **8.** Lewis *et al*, 1986; **9.** Mazzarella *et al*, 1990; **10.** Chaudhuri *et al*, 1990; **11.** Bennett *et al*, 1988; **12.** Edman *et al*, 1985; **13.** Lebeche *et al*, 1994; **14.** Pihlajaniemi *et al*, 1987; **15.** Baksh and Michalak, 1991; **16.** Michalak *et al*, 1992; **17.** Ozawa and Muramatau, 1993: **18.** Lucero *et al.*, 1994.

Table 1-1:

Selected lumenal proteins of the ER

M_r	**Name**	**Alias**	**Ca^{2+} binding capacity**	**C-terminal amino acid sequence**	**References**
100 000	Grp94	Endoplasmin ERp99/hsp108	10 mols Ca^{2+}/mol protein	KDEL	1-4
75 000	BiP	Grp78	Not determined	KDEL	5-7
60 000	ERp61	p5, Grp58 phosphatidylinositol specific phospholipase C	Not determined	QDEL	1, 8-11
58 000	PDI	ERp59/T3BP/GSBP	19 mols Ca^{2+}/mol protein	KDEL	12-13
55 000	CRT	See Table 1-2	>20 mols Ca^{2+}/mol protein	KDEL	15-16
44 000	RCB	Reticulocalbin	Not determined (but contains EF-hand Ca^{2+} binding sites)	HDEL	17
54 000	CST	ERcalcistorin/calstorin	> 20 mols Ca^{2+}/mol protein	KDEL	18

to be endoplasmin (glucose regulated protein 94 - Grp94), the immunoglobulin heavy-chain binding protein (BiP or Grp78), ER protein ERp61, protein disulfide isomerase (PDI, T_3-binding protein, or glycosylation site binding protein - GSBP), and calreticulin (Fig. 1-1B). Since this original observation by Koch's group, two additional Ca^{2+} binding proteins have been identified in the lumen of the ER - reticulocalbin (Ozawa and Muramatsu, 1993), the first ER Ca^{2+} binding protein containing the EF-hand high affinity Ca^{2+} binding motif (Kretsinger, 1988), and ERcalcistorin (Lucero *et al.*, 1994), a new Ca^{2+} binding protein abundant in the rat brain (Table 1-1). Recently, Ohsako reported the molecular cloning of an abundant male germ cell-specific Ca^{2+} binding protein of the ER, calmegin (Ohsako *et al.*, 1994; Watanabe *et al.*, 1994). This integral membrane 101 kDa protein was found to have amino acid sequence similarity to the P-domain of calnexin and calreticulin (Ohsako *et al.*, 1994). Taken together, the reticuloplasmins and other ER Ca^{2+} binding proteins may account for buffering of over 300 nmols Ca^{2+} per mg of protein (Macer and Koch, 1988). The luminal protein content of the reticuloplasm from plasmacytoma cells has been estimated by Koch (1987) to be around 30 - 100 mg/ml of which PDI and calreticulin account for 0.4% and 0.05%, respectively. The ER localization of all the reticuloplasmins have been confirmed by immunocytochemistry, their cDNA clones isolated, and the proteins characterized. BiP and PDI are now believed to have chaperone-like functions, capable of interacting with misfolded or unassembled proteins and assist them to assume their correctly folded conformation (Gething and Sambrook *et al.*, 1992). Grp94 is a glucose regulated protein that is activated when the cell is stressed (Hendrick *et al.*, 1993). ERp61 and ERp72 were found to be cysteine proteases involved in the degradation of incorrectly folded proteins within the ER (Urade *et al.*, 1993). The last member of the reticuloplasmins is calreticulin, a protein discovered 20 years ago as a high affinity Ca^{2+} binding protein of the SR membrane (Ostwald and MacLennan, 1974). It is now established that within non-muscle cells, one of the main Ca^{2+} sequestering protein is calreticulin (Milner *et al.*, 1991), binding up to 20 moles Ca^{2+} per mole of protein (Baksh and Michalak, 1992).

Calreticulin

Calreticulin (Fliegel *et al.*, 1989b; Smith and Koch, 1989; McCauliffe, 1990a) has been cloned and its amino acid sequence deduced from the cDNA isolated by Fliegel *et al.* (1989b). The cDNA encoding the rabbit protein of 418 amino acids has a molecular weight of 46 567 Da. Using cDNA fragments encoding either the NH_2- or carboxyl-terminus of calreticulin as hybridization probes, Fliegel *et al.* (1989b) showed hybridization to a 1.9 kb mRNA fragment in all the tissues tested: liver, kidney, brain, cardiac muscle, and fast- and slow-twitch rabbit skeletal muscle. With a longer period of exposure, a second band of approximately 3.75 kb could be visualized on the autoradiograms. A protein product corresponding to this higher mRNA species has not been found and if it does exist, it may represent expression of calreticulin under abnormal cellular conditions. The human calreticulin gene was isolated and localized to chromosome 19 (McCauliffe *et al.*, 1992). The gene was found to occupy ~ 6 kb of genomic DNA. Figure 1-2A describes

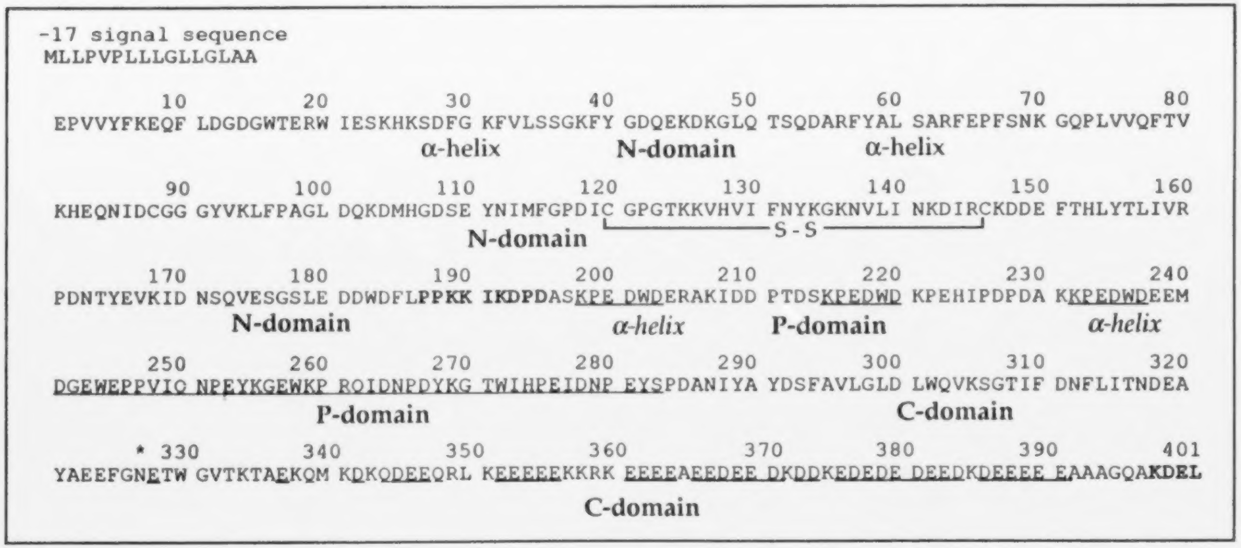
Figure 1-2: Amino acid sequence of rabbit skeletal muscle calreticulin

(A) The amino acid sequence of calreticulin is based on the amino acid sequence deduced from the nucleotide sequence of a cDNA clone encoding the skeletal muscle protein (Fliegel *et al.*, 1989b). N-domain (residues 1 - 170), P-domain (residues 187 - 285), and C-domain (residues 286 - 401) of calreticulin are labeled; the position of the disulfide bond is indicated by -S--S-; parts of repeat sequence A (the KPEDWD repeat sequence) within the P-domain are underlined; the long stretch of underlined sequences within the P-domain denotes the position of the repeat sequence B (see below); the bold sequence just after the N-domain represents the putative nuclear localization signal (NLS) - PPKKIKDPD; the asterisk at residue 327 indicates the possible N-glycosylation site; acidic residues within the C-domain are underlined; and the ER retention signal at the end of the C-domain is shown in bold (residues 398 - 401). The positions of the α-helical regions in the N-domain are indicated and α-helical regions within the P-domain are shown in italics and are labeled.

Adapted from Michalak *et al.*, (1992).

(B) Repeat sequences within the P-domain of calreticulin. Three repeat sequence A (numbered 1, 2, and 3) and three repeat sequence B (numbered 1, 2, and 3) are found within this domain of calreticulin. X denotes any amino acid. The consensus repeat pattern is shown. "-" denotes amino acid identity and numbers indicate amino acid positions within the rabbit skeletal muscle calreticulin sequence.

A



```
-17 signal sequence
MLLPVPLLLGLLGLAA

        10         20         30         40         50         60         70         80
EPVVYFKEQF LDGDGWTERW IESKHKSDFG KFVLSSGKFY GDQEKDKGLQ TSQDARFYAL SARFEPFSNK GQPLVVQFTV
                              α-helix     N-domain              α-helix

        90        100        110        120        130        140        150        160
KHEQNIDCGG GYVKLFPAGL DQKDMHGDSE YNIMFGPDIC GPGTKKVHVI FNYKGKNVLI NKDIRCKDDE FTHLYTLIVR
                            N-domain       |__________ S-S ____________|

       170        180        190        200        210        220        230        240
PDNTYEVKID NSQVESGSLE DDWDFLPPKK IKDPDASKPE DWDERAKIDD PTDSKPEDWD KPEHIPDPDA KKPEDWDEEM
           N-domain                       α-helix        P-domain                α-helix

       250        260        270        280        290        300        310        320
DGEWEPPVIQ NPEYKGEWKP RQIDNPDYKG TWIHPEIDNP EYSPDANIYA YDSFAVLGLD LWQVKSGTIF DNFLITNDEA
                P-domain                                           C-domain

     * 330        340        350        360        370        380        390        401
YAEEFGNETW GVTKTAEKQM KDKQDEEQRL KEEEEEKKRK EEEEAEEDEE DKDDKEDEDE DEEDKDEEEE EAAAGQAKDEL
                                          C-domain
```

B

REPEAT SEQUENCE A

Consensus PXXIXDPDAXKPEDWDE

1 188 – K K--K -----------S ------------------ 204

2 205 R A K--D-----T D S ----------------K 221

3 222 – E H--P----------- K ------------------ 238

REPEAT SEQUENCE B

G X W X P P X I X N P X Y X

1 242 —E ---E-----V--Q------E---K 255

2 256 ---E-----K--RQ---D------D---K 269

3 270 ---T-----I H----E--D------E---S 283

the amino acid sequence of rabbit skeletal muscle calreticulin as deduced from the nucleotide sequence of the cDNA obtained by Fliegel *et al.* (1989b). Comparison of the NH_2-terminal sequence of the purified protein and that obtained from cDNA analysis, revealed the presence of a 17-residue hydrophobic signal sequence that was removed during synthesis of calreticulin (Fliegel *et al.*, 1989b) (Fig. 1-2A). The presence of this signal sequence was confirmed by *in vitro* translation of mRNA encoding calreticulin (Fliegel *et al.*, 1989b). Hydropathy plots did not reveal potential transmembrane segments confirming that calreticulin is a peripheral membrane protein (Michalak *et al.*, 1991). Calreticulin terminated with the KDEL amino acid sequence (Fig. 1-2A) which corresponded to the ER retention signal (Munro and Pelham, 1987). Current opinion favors a model in which KDEL proteins are recognized and sorted in the Golgi region by binding to a 26 kDa non-glycosylated integral membrane KDEL-receptor in yeast, and a 76 kDa glycosylated receptor in mammals (Pelham, 1991). All resident ER proteins identified to date contain the KDEL ER retention signal or a variant of this amino acid sequence (Table 1-1).

Calreticulin Identities

Since its discovery over 20 years ago by Ostwald and MacLennan (1974), calreticulin has been rediscovered several times. Establishment of a central DNA and protein database in the 1980's allowed researchers easy access to established amino acid and nucleotide sequences. By the 1990's it became apparent that several laboratories were working with the same protein, but under different assumed names (Table 1-2). Calreticulin was

Table 1-2:

Calreticulin Identities

Name	NH_2-terminal amino acid identity to HACBP (15 amino acid window)	Source	Identified as a:
HACBP	100 %	Rabbit skeletal muscle	Ca^{2+} binding protein
Calregulin/CAB-63	100 %	Bovine liver	Ca^{2+} binding protein
CaBP3	80 %	Rat liver	Ca^{2+} binding protein
CRP55	80 %	Mouse plasmacytoma cells	Ca^{2+} binding protein
ERp60	73 %	Mouse plasmacytoma cells (MOPC-315)	ER cysteine protease
CRH1/CRH2	50%/55%	Barley	Ca^{2+} binding protein
Ro/SS-A	93 %	human Wil-2 cell line	SLE autoantigen
C1qR	93 %	human tonsil lymphocytes	C1q receptor
B50	77 %	Mouse melanoma	Melanoma antigen
Mobilferrin	73 %	Rat duodenal mucosa	Fe^{3+} binding protein
RAL-1	Incomplete NH_2-terminus	*Onchocerca volvulus*	Autoantigen
p407	54 %	*Aplysia californica* (marine mollusk)	long term memory molecule
p60 JSTX BP	100 %	*Nephilia clavata* (Joro spider)	Joro Toxin binding protein
Sm58	52 %	*Schistosoma mansoni*	*S. mansoni* antigen
p60 3'(+) SL binding protein	87 %	Vero 76 Cells (simian origin)	Rubella virus binding protein

Adapted from Kimberly Burns, Ph. D. dissertation, University of Alberta (1994) originally identified as the high affinity Ca^{2+} binding protein of the SR and hence was named HACBP. Calregulin/CAB-63 was isolated from bovine liver at about the same time calreticulin was sequenced and was found to have an identical amino acid sequence to calreticulin (Waisman *et al.*, 1985). CaBP3 (Peter *et al.*, 1992), CRP55 (Macer and Koch, 1988), ERp60 (Lewis *et al.*, 1985), and CHR1/CHR2 (Chen *et al.*, 1994) were all identified in their respective tissues as major Ca^{2+} binding proteins that could be important in the regulation of the respective physiological process being investigated. The remaining identities will be discussed in the section: "Non-ER/SR localization of calreticulin - A Clue to It's Biological Function?". The presence of several identical NH_2-terminal amino acid sequences identical to the HACBP of the SR prompted several laboratories studying ER and Ca^{2+} binding proteins to identify all these variant proteins with a common name. The name calreticulin was chosen to reflect both the protein's only known possible physiological function (**cal**cium binding) and localization within the cell, that is, the endoplasmic reticulum (**reticulin**) (Fliegel *et al.*, 1989b; Smith and Koch, 1989).

Characterization of Calreticulin

The amino acid sequence of rabbit calreticulin would predict a molecular weight of 46 567. However, when analyzed by SDS-PAGE (Laemmli system) calreticulin migrated with an apparent molecular mass of 60 000 - 63 000 (Waisman *et al.*, 1985; Milner *et al.*, 1991). Discrepancies between the predicted and observed mobilities on SDS-PAGE have been reported for several other Ca^{2+} binding proteins, including calsequestrin

(MacLennan *et al.*, 1983) and this discrepancy may be attributed to the high content of acidic residues within the protein sequence.

Calreticulin was originally isolated as a high affinity Ca^{2+} binding protein of the ER. The protein binds 1 mole Ca^{2+}/mole of protein with high affinity and over 20 moles Ca^{2+}/mole of protein with low affinity. The best characterized high affinity Ca^{2+} binding proteins such as calmodulin and troponin C contain an EF-hand consensus amino acid sequence (Kretsinger, 1988). This amino acid sequence forms a helix-loop-helix secondary structure responsible for coordinating the Ca^{2+} ion (Strynadka and James, 1989). Calreticulin does not contain the EF-hand amino acid consensus sequence, but it may contain the structural features associated with the helix-loop-helix that is responsible for high affinity Ca^{2+} binding. Localization of the high affinity site within calreticulin was one of the major goals of my work and it will be discussed later. Calreticulin also binds other ions including Zn^{2+} (Khanna *et al.*, 1986a, 1987) and Fe^{3+} (Conrad *et al.*, 1994). Conrad *et al.* (1994) documented that in addition to Fe^{3+}, calreticulin/mobilferrin binds other metals ($Zn^{2+} > Co^{2+} > Cu^{2+} > Pb^{2+}$), *albeit* with lower affinity than Fe^{3+}.

It is currently unclear whether calreticulin is glycosylated and/or phoshorylated. Whilst the amino acid sequence contains one potential glycosylation site (Asn-327: Asn-Xaa-Ser/Thr) (Fig. 1-2A), this site is only utilized in certain species of calreticulin (Waisman *et al.*, 1985; Van *et al.*, 1989). The glycosylation site has been conserved in the calreticulins from such diverse species as amphibians and mammals (Michalak *et al.*, 1992). The conservation of such a site over millions of years of evolution would therefore suggest that it may be relevant to the protein's function.

However, no carbohydrate has been detected in either skeletal of smooth muscle calreticulin (Milner *et al.*, 1991), nor in human (McCauliffe *et al.*, 1990b) or murine calreticulins (Lewis *et al.*, 1985). Chicken and rabbit liver calreticulin also contain no carbohydrate residues (Waisman *et al.*, 1985). Despite the absence of glycosylation of the aforementioned calreticulin species, both bovine liver and bovine brain calreticulin have been shown to be glycosylated (Matsuoka *et al.*, 1994). Glycosylation was demonstrated by calreticulin's binding to Concanavalin A-agarose and it's sensitivity to endoglycosidase H digestion (Khanna *et al.*, 1987; Lewis *et al.*, 1985; Matsuoka *et al.*, 1994). The bovine brain protein was found to contain a high mannose sugar structure with the composition of $(GlcNAc)_2Man_5$ (Matsuoka *et al.*, 1994) which is typical of a resident ER protein. In this species, however, the glycosylation was on Asn-162 instead of Asn-327. In other calreticulins, an aspartic acid is at position 162 instead of an asparagine. The Asn-162 within the brain form of calreticulin is flanked by long stretches of conserved sequences that are identical to other known calreticulin sequences. The N --> D change at amino acid 162 in bovine brain calreticulin species may thus represent a species-specific change that may be reflected in the physiological function of the protein in the brain. In addition, Khanna *et al.* (1987) also showed that a population of bovine liver calreticulin (0.5% - 1%) was non-glycosylated and could be resolved from the glycosylated calreticulin by Concanavalin A-agarose affinity chromatography. A detailed carbohydrate analysis is thus required of all calreticulins that are isolated to establish firmly the glycosylation patterns of the protein and the precise trafficking of this protein between different cellular compartments.

Calreticulin has potential consensus sites for phosphorylation by casein kinase II and protein kinase C (Michalak *et al.*, 1992; Singh *et al.*, 1994). Whether these sites are utilized by all variant forms of calreticulins remains to be properly documented. Recently, calreticulin was found to interact with the 3' Cis-acting element of the rubella virus RNA molecule as a phosphoprotein (Singh *et al.*, 1994). These authors documented that viral infection of Vero 76 cells promoted the phosphorylation of simian calreticulin and they also showed autophosphorylation of simian calreticulin (Singh *et al.*, 1994). Singh *et al.* (1994) also reported that there are at least three forms of calreticulin in Vero 76 cells but only one form of the protein was phosphorylated. At present it is not clear how much of total cellular calreticulin may exist in the phosphorylated form or what proportion of the phosphorylated form is bound to the rubella viral RNA molecule. Phosphorylation, as with glycosylation, is very species-specific and appears to be enhanced during viral infection. Both glycosylation and phosphorylation of canine pancreatic calreticulin will be discussed in detail in "Chapter Three: Purification, expression, and biochemical characterization of calreticulin and calreticulin domains".

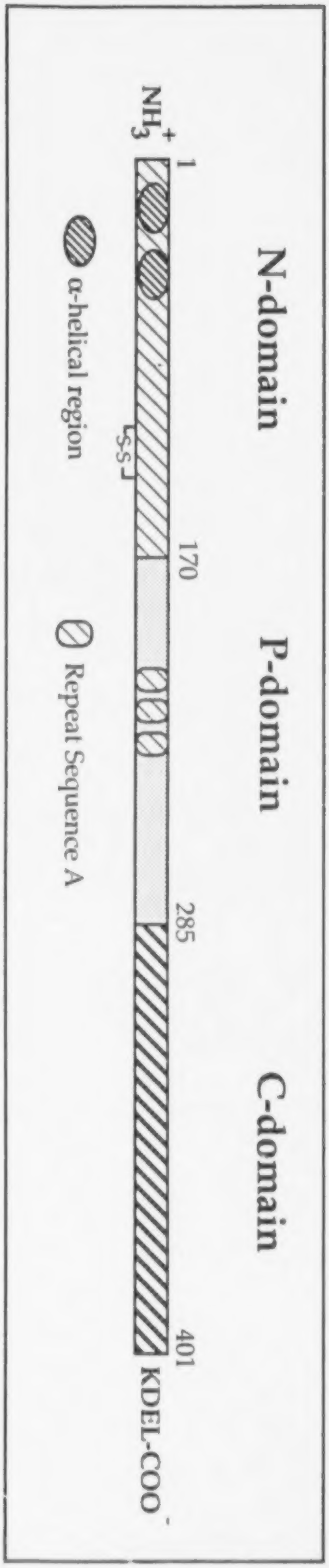
Domain structure of Calreticulin

Calreticulin has been proposed to be divided structurally into distinct domains (Fliegel *et al.*, 1989b; Smith and Koch, 1989) (Fig. 1-2A and Fig. 1-3). The first domain, which encompasses the NH_2-terminal half of the molecule (residues 1-170) is predicted to have a globular structure. The NH_2-terminus of the mature form of the protein is

Figure 1-3: The domain-like structure of calreticulin

Calreticulin has been proposed to be divided structurally into distinct domains (Fliegel *et al.*, 1989b; Smith and Koch, 1989). The first domain, the **N-domain**, is predicted to contain 8 anti-parallel ß-strands connected by two regions of short α-helices (indicated by ovals). This region leads into a proline-rich **P-domain** containing repeat sequence A (PXXIXDPDAXKPEWDWE) repeated three times (identified by rectangles) and repeat sequence B (GXWXPPXIXNPXYX), also repeated three times (located just after the rectangles). The carboxyl-terminal quarter of the protein (the **C-domain**) is highly acidic containing a large amount of negative charges. The position of the intramolecular disulfide bond within the N-domain is shown. Numbers indicate amino acid positions within the rabbit skeletal muscle calreticulin sequence (Fig. 1-2A).

Modified from Baksh and Michalak (1991).


N-domain
P-domain
C-domain
NH_3^+
1
170
285
401
KDEL-COO⁻
S-S
α-helical region
Repeat Sequence A

predicted to contain 8 anti-parallel ß-strands connected by protein loops (N-domain). Two regions of short α-helices (indicated by ovals in Fig. 1-3) are predicted within residues 1 - 74 of rabbit skeletal muscle calreticulin (Fliegel *et al.*, 1989b). This region leads into a proline-rich sequence (P-domain) containing 16 mole % prolines spaced every four or five amino acids (from residue 187 - 285). The first part of this region is highly charged and contains the sequence PXXIXDPDAXKPEWDWE (identified as repeat sequence A and by rectangles in Fig. 1-3) which occurs at residues 188 - 204 and repeats from residues 205 to 221 and 222 to 238 (Fliegel *et al.*, 1989b) (Fig. 1-2B). This is followed by a second repeat sequence GXWXPPXIXNPXYX (identified as repeat sequence B and positioned directly after repeat sequence A in Fig. 1-3) which occurs at residues 242 to 255, 256 to 269, and 270 - 283 (Fig. 1-2B). This is followed by a proline-, serine-, and threonine-rich sequence (residues 246 - 316) which is predicted to contain a repeating, rigid turn structure separating the globular head of the protein from the acidic tail. The carboxyl-terminal quarter of the protein (the C-domain) is highly acidic containing a large amount of negative charges. In the last 57 residues of the C-domain, 37 are aspartic or glutamic acid (Fliegel *et al.*, 1989b; Smith and Koch, 1989; McCauliffe *et al.*, 1990b; Murthy *et al.*, 1990). The C-domain also terminates with the KDEL ER retention signal. The functional implications of these distinct domains within calreticulin are not clear are present but studies by various researchers over the past 20 years have led to the hypothesis that calreticulin is a multifunctional protein performing distinct functions in different regions of a variety of eukaryotic cells.

The Amino Acid Sequence of Calreticulin is Highly Conserved

There is a high degree of amino acid sequence conservation among different calreticulin sequences. Figure 1-4 is a comparison of calreticulin amino acid sequences from rabbit to *Drosophilia*. Rabbit, mouse, human, rat, and *Aplysia* calreticulin's share over 90 % amino acid identity within their amino acid sequence. The *Drosophilia* calreticulin sequence is available and we observe over 80 % similarity between the two amino acid sequences. This list is by no means exhaustive. Missing from this list is plant calreticulin, CRH1/CRH2, which was found to be highly cross-reactive against calreticulin antibodies (Chen *et al.*, 1994). Also not shown is calreticulin's similarity to the integral ER membrane chaperone calnexin (Tjoelker *et al.*, 1994). Calnexin is a 90 kDa high affinity Ca^{2+} binding protein that functions to guide newly synthesized proteins that have translocated across the ER barrier (Bergeron *et al.*, 1994). Calnexin has been isolated in association with major histocompatability complex (MHC) class I molecules, T-cell receptor, and membrane immunoglobin (Hochstenbach *et al.*, 1992). Calnexin is thought to promote the proper assembly of protein complexes during transit through the ER. The translocation and interaction of such complexes has been found to be Ca^{2+} dependent (Sambrook, 1990). It is not surprising that when the sequence of calnexin became available, calnexin and calreticulin shared the highest degree of similarity to the P-domain and C-domain regions of calreticulin - regions that are involved in high affinity and high capacity Ca^{2+} binding in calreticulin (Fig. 4-10). A detailed analysis of the Ca^{2+} binding domains within calreticulin and the similarity to calnexin will be discussed in "Chapter Four: Identification of the Ca^{2+} binding domains

Figure 1-4: Comparison of amino acid sequences of different calreticulins

The amino acid sequences of calreticulins were taken from Fliegel *et al.*, (1989b) for rabbit calreticulin; McCauliffe *et al.*, (1990a) for human calreticulin; Smith and Koch, (1988) for mouse calreticulin; Murthy *et al.*, (1990) for rat calreticulin; Kennedy *et al.*, (1992) for *Aplysia* calreticulin; Treves *et al.*, (1992) for *Xenopus* calreticulin; Hawn *et al.*, (1993) for *S. mansoni* calreticulin; Unnasch *et al.*, (1988) for Onchocercal calreticulin; Smith *et al.*, (1992) for C. elegans calreticulin; and McCauliffe *et al.*, (1990a) for *Drosophila* calreticulin. (:) represents perfect matches and (-) represents a gap. *On*, Onchcercal calreticulin.

Adapted from Nash *et al.*, (1994).

```
               -17               10         20         30         40         50
Rabbit         MLLPVPLLLGLLGLAAA EPVVYFKEQF LDGDGWTERW IESKHKSDFG KFVLSSGKFY GDQEKDKGLQ
Human          :::S:::::::::::.: :::::::::: :::::::S:: :::::::::: :::::::::: ::E:::::::
Mouse          ::::::::::::::::: .::.:::::: ::::A::N:: .::::::::: :::::::::: ::L:::::::
Rat            :::S::::::::::::: .:..:::::: ....A..N:: .::::::::: :::::::::: ::.:::::::
Aplysia        :K-V:-::CA:::.:.: .:T:V:::E: G:-:-:A::: .::::::::.: ::::.A:::: ::A:::::.:
X. laevis - 1                    ::::::::E: -::::::Q:: ..::::.:Y: ..K..A.... ::.:::::::
X. laevis - 2                               :::::::Q:: .:::::::Y: ::K::A:::: ::.:::::::
S. mansoni     :-:SI::T:-::SKY:L GHEVW:S:T: -P...-I:N: .Q:TYNA.KQ GEFKVEAGK. PVDPI.DLGL
Onchocercal        incomplete NH2 end                                        ::AV:::::K
C. elegans     :K-S--:C:-::::::: S.E:::::E: N:AS.EKK:: .Q::::D::: A:K::.:::F DVES.:.:.:
Drosophila         partial sequence

                       60         70         80         90        100        110        120
Rabbit         TSQDARFYAL SARFEPFSNK GQPLVVQFTV KHEQNIDCGG GYVKLFPAGL DQKDMHGDSE YNIMFGPDIC
Human          :::::::::: ::S::-:::: :::T:::::: :::::::::: :::::::N.: ::T::::::: ::::::::::
Mouse          :::::::::: ::.::-:::: :::::::::: :::::::::: :::::::S:: :::::::::: ::::::::::
Rat            :::::::::: :::::::::: ::T::::::: :::::::::: :::::::G:: :::::::::: ::::::::::
Aplysia        :::::::::: ::.:.K:::E :KT::.:::: :::::::::: ::::.:.SD: ::S::::.:P ::::::::::
X. laevis - 1  :::::::::. :S::.S:::: D:T:::::.: :::::::::: ::::::::A: .:T.:::.:: ::::::::::
X. laevis - 2  :::::::::. :S:::S:::: D:T::.::.: :::::::::: ::::::::D: .:T.:::.:: ::::::::::
S. mansoni KT::::::::G.: :KIS:T:::. :KT.:.:::: F.K.V:::: A:I:::GS D. :P:K.::.:P :K::::::::
On             :.:::.::S. .:::.KS::: ::KS::I::S :::::D:::: ::::::..:D. NLE:S::..P :H::::::::
C. elegans     :::::.::SR A:.:.K:::: :KT::.:.:: ::::.::::: :::::..R:D. :LG:.::..P ::.:::::::
Drosophila                       ::::::.: :::::::::-A ::::::DC:: :::::::

                       130        140        150        160        170        180        190
Rabbit         GPGTKKVHVI FNYKGKNVLI NKDIRCKDDE FTHLYTLIVR PDNTYEVKID NSQVESGSLE DDWDFLPPKK
Human          :::::::::: :::::::::: :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
Mouse          :::::::::: :::::::::: :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
Rat            :::::::::: :::::::::: :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
Aplysia        :::::::::: ::::::::.:. K::::::::V :.:::::::: :::::::::: :EK.:::D:: A::::::A:T
X. laevis - 1  ::P::::::: :.::K::.Q: .::::::::.S:::::::::: :::::::::: ::K::::::: ::::::::::
X. laevis - 2  ::P::::::: :Q::K::.Q: :::::::::S :::::::::: :::::::::: ::K::::::: .:::::::::
S. mansoni     :.A::::::: :::::::H:: K:.:P::::L K::::::::N :N:K:::L.: :AK::E:::: ::::.:::::
On             :::::::::: :H::D.:H.: K::::::::V :::.:::::N S::::F:Q:: .FK.:::E:: A:::::::::
C. elegans     ::-:..:::: .::::E:K:: K:.:T::S:: .::::::::L S::::::::: .ES.Q.:::: E::L::::::
Drosophila                                                   ::.L:: :EK:::N:: :::::.:::

                       200        210        220        230        240        250        260
Rabbit         IKDPDASKPE DWDERAKIDD PTDSKPEDWD KPEHIPDPDA KKPEDWDEEM DGEWEPPVIQ NPEYKGEWKP
Human          :::::::::: :::::::::: :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
Mouse          ::::::A::: :::::::::: :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
Rat            ::::::A::: :::.:::::: :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
Aplysia        :P::::K::. :::::E:::: :D:.:::::: :::::::::.: :::::::::: ::::::::.:D ::::::::::
X. laevis - 1  :::::.:K::. .::::.:::: :E:K:::::. :::::::::: V::::::::: :::::::::: ::::::::::
X. laevis - 2  :::::.:K::. ::::P:::: :E:K:::::. :::::::::: V::::::::: :::::::::: ::.LQ:::::
S. mansoni     :D::NDK::. ::V.EQF::: :D:K::.N:: Q:KT:::N:: :::.:::.A: :::::R:QKD ::::::::T:
On             ::::::K::: :::::EF::: ED:K:::::: :::::::::: :::::::.:: ::::::::..D ::::::::::
C. elegans     ::::::K::: :::::EY::: .E:A:::::. :::::::::: :::::::.:: :::::::.:D ::::::::::
Drosophila     ::::T:.::: :::

                       270        280        290        300        310        320        330
Rabbit         RQIDNPDYKG TWIHPEIDNP EYSPDANIYA YDSFAVLGLD LWQVLSGTIF DNFLITNDEA YAEEFGNETW
Human          P::::::::: :::::::::: :::::..::: ::.:G::::: ::::K::::: :::::::::: ::::::::::
Mouse          P::::::W:: :::::::::: :::::::::: :::::::::: ::::K::::: :::::::::: ::::::::::
Rat            :::::::::: :::::::::: :::::::::: :::::::::: ::::K::::: :::::::::: ::::::::::
Aplysia        .:.::::::: K..::::::: ::E.:DK.:S FAD:G..:.: ::::KA:::: ::V:::DSVE ::::::::::
X. laevis - 1  :::::::::: K::::::::: ::.::D..:S ::::G::::: ::::K::::: :::::....K H:::Y:::::
X. laevis - 2  :::::::::: K:::::.::: ::.::ST.:S :.::G:I::: ::::K::::: ::::.::::K ::::Y:::::
S. mansoni     :R::::K::: E:K:VQ:::: ::KH:.E.:. LND.GY.:.: ::::D::.:: ::.:::DSPD F:--------
On             K:KK::AW:: K::::::.I: .:.::D:.:. ::D.G..:.: :::::::::: :D...:DSVE E:KK::EK..
C. elegans     K::K::A::: K:::::E::: ::.::DE.:S :.:.G..:.: ::::K::::: ::..::DSVE E:::HAA::.

                       340        350        360        370        380        390        401
Rabbit         GVTKTAEKQM KDKQDEEQRL KEEEEEKKRK EEEEAEEDEE DKDDKEDEDE DEEDKDEEEE EAAAGQAKDEL
Human          :::::::::: :::::::::: :::::::::: :::::::.K:. :-.::....: ::::.:.:: :D..:::KDEL
Mouse          :::::::::: :::::::::: :::::::::: :::::::.K:. :-........ .:..:.:.:: .S-.:::KDEL
Rat            ::::A::::: :::::::::: ::::.::::: ::::::K..: :D.:-::::: ...-:.:.:: .:-T:::KDEL
Aplysia        :K::D.::K: ::A::::D:K AK:::::::: :::.:NK:.: .-..A:.:.: .:.:------ .::.E--KDEL
X. laevis - 1  ::::E:::K: :.Q::::D:K :Q:::::T:: :::PQ::..: :D::E:.K.: .K:.E.::.: T--.-L-KDEL
X. laevis - 2  ::::E:::K: :.QQ:::D:K :Q:.::N:Q: :::PQ::..- --::E:.K.:K.::.E..:: -T--.-:-KDEL
S. mansoni     -------------------K E:G:RLW...Y.A:V:K:QSS A:::::.A.:TK:RKELPY.AKACDDP.GDHDEL
On             KKI:REG::K -:G:KTKK:K -:K:KNE:I: :KMKKRKRAN R-KKKK
C. elegans     DKL::.::E: :.:A:::T:K A:::A-.:.A -:::K-.:.- ------.:-- ----:..-.. ----:---HDEL
```

within calreticulin".

A high degree of conservation in the amino acid sequences of the proteins in Fig. 1-4 is observed within the hydrophobic N-domain and the proline-rich P-domain of these various calreticulins. High sequence variation is, however, seen within the highly acidic C-domain of the protein. Divergence is specifically observed in the Onchocercal Ral-1 protein (On in Fig. 1-4) which has a basic carboxyl tail as compared to calreticulin's highly acidic tail (McCauliffe *et al.*, 1990b). Patients that show symptomatic features of onchocerciasis or river blindness have strong antigenic reaction to this protein (Rokeach *et al.*, 1991). Another difference between the Onchocercal protein and calreticulin is that the former does not terminate with the KDEL ER retention signal (Unnasch *et al.*, 1988), suggesting that the Onchocercal protein may escape the ER and be secreted. This presence of a variant carboxyl-terminal tail may represent a divergent function of calreticulin in the pathogenesis of onchocerciasis. Similarity, however, in the amino acid sequences of the remainder of the calreticulin species indicate that calreticulin is a ubiquitous eukaryotic protein carrying out an important function within the cell.

Distribution of Calreticulin

Calreticulin (referred to as calregulin) was quantified by Khanna and Waisman (1986) using a radioimmunoassay in a variety of bovine tissues. It was shown that calreticulin was found in high amounts in pancreas (~ 540 μg/g of tissue), liver (~ 375 μg/g of tissue), and the testis (~ 256 μg/g of tissue). Canine pancreatic tissue was shown to contain

~ 500 μg/g of tissue calreticulin (Baksh *et al.*, 1992). Lower quantities of calreticulin were found in the kidney, spleen, adrenals, and parathyroid (~ 100 μg/g of tissue). The cerebral cortex had the lowest amounts (~ 20 μg/g of tissue). The bovine erythrocyte was the only cell shown to not contain calreticulin (Khanna and Waisman, 1986) and the only eukaryotic cell not to contain detectable amounts of calreticulin was yeast.

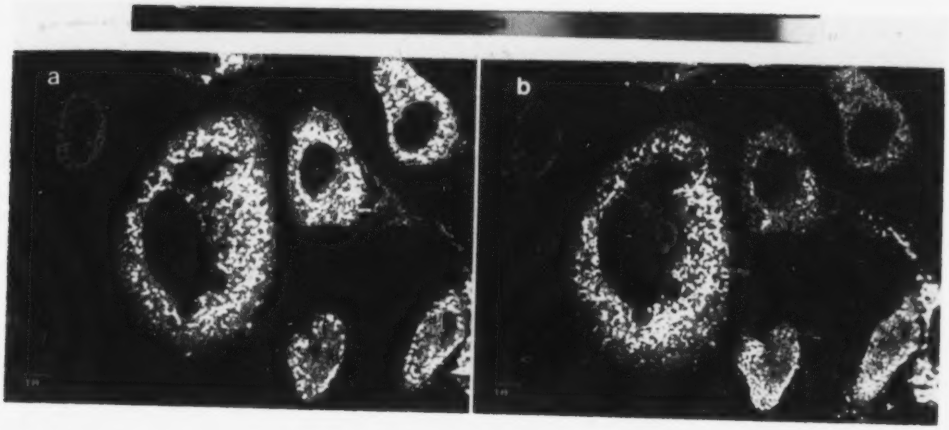
ER/SR Localization of calreticulin

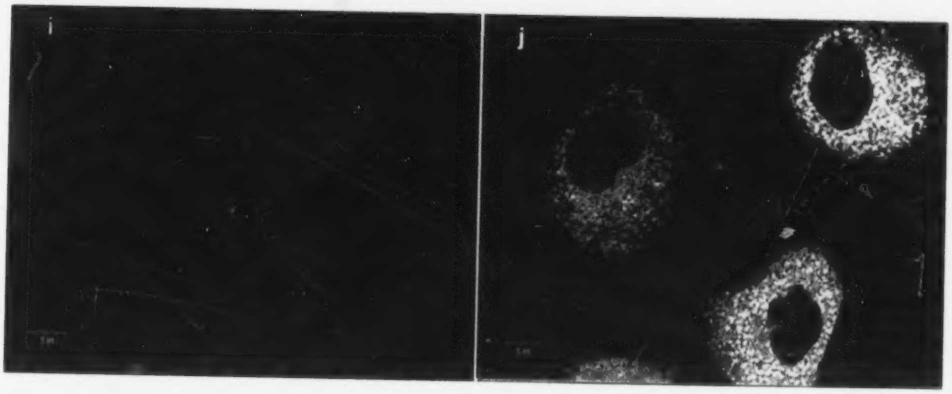
Calreticulin contains the ER retention signal KDEL. This signal is thought to retain the protein within the membranous barriers of the ER/SR. Indirect immunofluoresence staining of frozen sections and cultured cells from a variety of tissues showed that calreticulin localizes predominantly to the junctional SR and T-tubule areas in skeletal muscle, to the SR in smooth and cardiac muscle cells, and to the ER in a variety of non-muscle cells including uterine muscle, fibroblasts, intestinal epithelium, and cardiac endothelium (Fliegel *et al.*, 1989c). Using confocal fluorescence microscopy, Michalak *et al.* (1991) examined the distribution of calreticulin and calsequestrin, in relation to the ER, in pancreatic cells grown in culture. They identified calreticulin, and not calsequestrin, as the major Ca^{2+} sequestering protein present in rough pancreatic microsomes (Fig. 1-5). Calreticulin distribution within pancreatic acinar cells (Fig. 1-5, a) corresponded to the ER arrangement as visualized with TRITC-con A (Fig. 1-5, b). TRITC-con A stained specifically to mannose residues on glycoproteins within the ER network. Calsequestrin, however, was not detected in pancreatic acinar cells

Figure 1-5: Confocal microscopy of pancreatic cells in culture after immunolabeling with specific antibodies against either calreticulin, calsequestrin or TRITC-con A.

(a, b): A group of pancreatic acinar cells fixed in formaldehyde, permeabilized and double labeled with anti calreticulin (a) and TRITC-con A (b). Pattern of calreticulin distribution in these cells closely resembles distribution of ER as visualized with con-A (b).

(i, j): Confocal fluorescence microscopy of pancreatic acinar cells after double labeling with antibodies against calsequestrin (i) and TRITC-con A (j). Diffuse anti-calsequestrin staining (i) is observed that approximates to background levels and this would not be discernible at all if these images were not color coded. The ER arrangement in the calsequestrin-stained cells is shown in (j). Substitution of specific antibodies against calreticulin and calsequestrin with respective non-immune goat and rabbit sera, respectively gave no specific staining. Omission of primary antibodies from the staining procedure gives no specific staining with either anti-goat or anti-rabbit secondary antibodies. Scale bars are calibrated in micrometers. The color bar above plaques a and b shows the color attributes for grey levels 0-255.


a
b


i
j

(Fig. 1-5 i), even though the cells still showed characteristic ER staining as visualized by TRITC-con A staining (Fig. 1-5, j). They convincingly showed that calreticulin is localized to the ER in pancreatic acinar cells. Using deletion mutants of calreticulin, Sonnichsen *et al.* (1994) showed that the KDEL tetrapeptide sequence at the carboxyl-terminus and the high density of negative residues adjacent to the KDEL sequence were required for retention of calreticulin within the ER. Deletion of these elements led to 28% increase in the secretion of calreticulin from the ER, thus supporting their hypothesis of the importance of the KDEL signal, and it's adjacent Ca^{2+} binding site, to the retention of calreticulin within the ER.

Non-ER/SR localization of calreticulin - A Clue to It's Biological Function?

Nuclear localization

Numerous immunocytochemical staining of calreticulin confirmed the ER localization of calreticulin (Michalak et *al.*, 1991; Tharin *et al.*, 1992: Milner *et al.*, 1991). In some cell types, calreticulin can also be detected in the nuclear envelope and in the nucleoli (Michalak *et al.*, 1992). Calreticulin does contain a putative nuclear localization signal (NLS) within the P-domain (Fig. 1-2A, residues 188 - 196). Whether this NLS is important for calreticulin's trafficking is still an open question. Opas *et al.* (1991) have shown that when myoblast fusion is inhibited in proliferating rat L6 cells, these cells lose their nucleoli staining but still retain the ER staining. In differentiated (that is, fused) myotubes both the

ER and the nucleolar staining of calreticulin are abolished (Fliegel *et al.*, 1989a). The reason for calreticulin staining of nucloeli is still unclear at present. Nuclear envelope staining is not surprising since the ER is continuous with the outer nuclear membrane. However, it remains unclear why this pattern of staining is not universally seen.

What is calreticulin doing in the nucleus? A function for nuclear calreticulin was found when Burns *et al.* (1994) and Dedhar *et al.* (1994) showed that calreticulin interacted with glucocorticoid and androgen steroid receptors, respectively to inhibit their DNA binding abilities to their respective steroid response elements in the nucleus. Calreticulin was found to bind to the sequence, KVFFKR, present between the two Zn^{2+} finger structures within the sequence of these steroid receptors. By binding to this peptide sequence, calreticulin prevented the interaction between the glucocorticoid and androgen receptors with their respective response element and hence prevented the transcription of these genes (Burns *et al.*, 1994; Dedhar *et al.*, 1994). How can calreticulin gain access to the steroid receptors? The glucocorticoid and the androgen receptors are thought to shuttle between the nucleus and cytoplasm to modulate the transcription of the receptor gene. The nuclear localization observed by Opas *et al.* (1991) would lend support to the association of calreticulin and the glucocorticoid receptor; but a mechanism of how these elements, located in areas that are physically separated from each other, contact and associate together remains to be determined.

Cytoplasmic localization of Calreticulin

Calreticulin has been suggested to be one of four proteins that are part of the Ro/SS-A antigenic complex present in the majority of patients with Sjorgren's syndrome, with systemic lupus erythematosus (SLE) and congenital complete heart block of neonates (Lux *et al.*, 1992). The Ro/SS-A antigenic complex is composed of these four proteins and a cytoplasmic RNA molecule - the hY RNA (Sontheimer *et al.*, 1994). One of the immunoprecipitated proteins with the lupus antiserum had the NH_2-terminal amino acid sequence of calreticulin (Deutscher *et al.*, 1988) and was later referred to as human calreticulin. Based on this observation, the authors classified the protein as a Ro/SS-A autoantigen. Recombinant calreticulin, however, was not recognized by antibodies against SLE patients and in certain cases, anti-calreticulin antibodies have failed to immunoprecipitate the Ro/SS-A RNA (Rokeach *et al.*, 1991). Thus it is still debatable whether or not calreticulin is a part of the Ro/SS-A antigenic complex.

Calreticulin was identified by Yokoi *et al.* (1993) as autoimmune antigens in Long Evans Cinnamon rats (LEC), rats that show spontaneous hereditary hepatitis and hepatic carcinoma. Calreticulin autoantibodies have been detected only in LEC patients and in normal patients with drug-induced hepatitis (Yokoi *et al.*, 1993). Satoh *et al.* (1989) demonstrated that patients with halothane-induced hepatitis carry serum antibodies to novel liver microsomal antigens, suggesting that these antigens may play an immunopathological role in the development of liver damage. Both calreticulin (TFA 63kDa) and another ER resident protein, protein disulfide isomerase (TFA 57 kDa), were identified as halothane-induced hepatitis antigens. Rokeach *et al.* (1991) also observed the presence of autoantibodies to calreticulin in the sera of patients with onchocerciasis. These studies show that antibodies to calreticulin and PDI must have easy access to their respective antigens resulting in the autoimmune response. How these ER specific proteins get out of the ER and interact with serum factors still remains a debatable topic. Continued investigation of the roles these microsomal antigens play in the production of autoimmune antibodies and contribution to various disease process may shed some light into calreticulin's role in autoimmunity.

Calreticulin and PDI were found in the cytosol from homogenates of mammary glands prepared under mild conditions (Ghosal *et al.*, 1994). Ghosal *et al.* observed an increase in the levels of both calreticulin and PDI during lactation. Furthermore, they found an association of calreticulin, PDI and BiP with intracellular lipid droplets of milk lipid globules. But, this association was found to be transient indicating that these proteins may be lost from the intracellular lipid droplets before or during their secretion as milk lipid droplets (Ghosal *et al.*, 1994). What controls this association? Does Ca^{2+} binding to calreticulin have an influence in this process? The only precedence for a Ca^{2+}-dependent association of a soluble protein with a lipid moiety is the annexin family of Ca^{2+} binding proteins (Burgoyne *et al.*, 1990). These are non-EF hand Ca^{2+} binding proteins that associate with membrane phospholipids in a Ca^{2+}-dependent manner. This transient association between calreticulin and milk lipid droplets will have to examined in greater detail and may help calreticulin researchers to develop an understanding for the physiological importance of this molecule.

A 56 kDa protein was discovered by Conrad *et al.* (1990) when they searched for iron binding proteins in the duodenal mucosa of rats. This 56 kDa protein, identified as mobilferrin was later shown to have an identical NH_2-terminal sequence to calreticulin (Conrad *et al.*, 1993a). Antibodies to mobilferrin cross-reacted with calreticulin and antibodies to calreticulin cross-reacted with mobilferrin, further confirming the identity of these two proteins. The complete amino acid sequence of mobilferrin has not been determined nor is the location of the iron binding site. Mobilferrin is of cytoplasmic origin and further adds confusion to the ER specific localization of calreticulin. These results illustrate that calreticulin may escape the ER retrieval/retention system and find its way to the cytoplasm to carry out its varied functions.

Another cytoplasmic twist to calreticulin's ER localization was observed when it was shown that calreticulin bound to the cytoplasmic domain of the α-subunit of the communication molecules within the cell - the integrins (Rojiani *et al.*, 1991). This subunit contained the amino acid sequence KLGFFKR, a sequence that was very similar to the calreticulin binding amino acid sequence within steroid receptors - KVFFKR. The functional significance of these findings still remains to be elucidated. Calreticulin may bind to the α-subunit and promote the interaction between the adjacent β-subunit, producing a rearrangement of the chains. This may transduce into movement of these chains on the extracellular face and thus stimulating a signal (Williams *et al.*, 1994).

Calreticulin's role in this process is still being investigated, but it adds to the growing list of non-ER localization of this protein.

Both resting, mouse and human T-lymphocytes contain very low levels of calreticulin mRNA and protein (Burns *et al.*, 1992). Mouse splenocytes stimulated with concanavalin A exhibited an induction in calreticulin mRNA and a 5-fold increase in the immunoreactive calreticulin protein band (Burns *et al.*, 1992). These results clearly demonstrate the presence of the ER specific Ca^{2+} binding protein in activated T-lymphocytes. In T-lymphocytes calreticulin has been found in the lumen of lytic granules in association with perforin (Dupuis *et al.*, 1993). Perforin is involved in the killing function of cytotoxic T-lymphoctyes and requires Ca^{2+} to become activated (Gardner, 1989). Dupuis *et al.* (1993) proposed that calreticulin might regulate the levels of free Ca^{2+} within cytotoxic granules thereby regulating the activation of perforin. The role of calreticulin in this potent killing process remains to be investigated.

Another granular location of calreticulin was reported by Nakamura *et al.* (1993) when they were looking for proteins involved in sperm-egg fusion. These authors observed the presence of calreticulin in spermatocytes, spermatids, Seritoli cells, and in the acrosome vesicles. The acrosome calreticulin was not glycosylated, and had comparable Ca^{2+} binding abilities to rabbit skeletal calreticulin, and was found to contain the KDEL retention signal (Nakamura *et al.*, 1993). The acrosome arises from the Golgi apparatus of the spermatid as it differentiates into a spermatozoon (pp. 94-96, Hafez, 1982). This organelle contains several hydrolytic enzymes, including hyaluronidase and acrosin, which are necessary for fertilization (pp. 94-96, Hafez, 1982). Nakamura *et al.* (1993) proposed that calreticulin is incorporated into the acrosomal vesicles *via* the Golgi apparatus during spermatogenesis. Acrosomal vesicular formation is an important event prior to fusion with the recipient egg cell and presence of both Ca^{2+} (Yanagimachi *et al.*, 1974) and Zn^{2+} (Clapper *et al.*, 1985) was shown to be important for sperm-egg fusion to occur. Thus calreticulin's role might be similar to it's role in cytotoxic T-lymphocytic granules - providing Ca^{2+} for specific physiological processes. Again, as with the non-ER localizations of calreticulin, the protein must have to bypass the retrieval/retention system of the ER and escape into these granules.

Calreticulin was also found to localize to the focal activation sites of neutrophils undergoing phagocytosis (Stendahl *et al.*, 1994). The process of phagocytosing microorganisms is believed to require Ca^{2+} (Valerius *et al.*, 1981), and both calreticulin and SERCA2b's cellular distribution pattern were found to change to a ring-like localization around the phagosome after injestion of yeast particles (Stendahl *et al.*, 1994). Stendahl *et al.* (1994) postulated that the physiological role of Ca^{2+} store accumulation during phagocytosis may be to generate subcellular $[Ca^{2+}]$ gradients that are responsible for controlling Ca^{2+} sensitive cellular activation events around the vicinity of the neutrophil. By having this localized concentration of stored Ca^{2+} (provided by the presence of calreticulin?), the cell would be able to regulate its Ca^{2+} response through a relatively low number of Ca^{2+} storage organelles concentrated at the site of Ca^{2+} action, rather than through an excess of uniformly distributed Ca^{2+} stores. How calreticulin associates with the neutrophils or how it localizes to the site of phagocytosis still has to be determined.

Secretion of Calreticulin

A small percentage of calreticulin has been found in normal human plasma. While purifying protein Z, a vitamin K-dependent glycoprotein, Sueyoshi *et al.* (1991) discovered that it copurified with calreticulin. Furthermore, they established that calreticulin was present at levels of ~ 60 ng/ml of human plasma. This observation supported the potential for the generation of circulating antibodies to calreticulin observed in SLE and LEC patients. Further support for the secretion of calreticulin came from studies by Kuwabara *et al.* (1994). They were investigating vitamin K-dependent coagulation factors and their affect on coagulation events at endothelial membrane surfaces. In the course of this study, a 55 kDa protein was purified to homogeneity from bovine lungs that had an identical NH_2-terminal amino acid sequence as rabbit skeletal calreticulin. Calreticulin bound to Factor IX/IXa in a dose-dependent and saturable manner and was found to bind to endothelial cells at a K_d of ~ 7.9 nM. Furthermore, it was shown that the C-domain of calreticulin bound to Factor IX and that this domain of calreticulin could act as a very strong anticoagulant preventing coronary thrombosis in the canine model used. It thus appeared that even though a small population of calreticulin is present in the blood, its effects are potent and it remains to be shown what other effects calreticulin may have when not in its normal location of the ER.

As mentioned earlier, calreticulin has also been shown to bind to the 3'(+) stem-loop structure of the rubella viral RNA molecule (Singh *et al.*, 1994). Rubella virus is the causative agent of German measles and has been shown to cause human fetal death in infective individuals (Wolinsky, 1990). It was found that calreticulin bound to rubella viral RNA as a phosphoprotein and that infection with the virus led to a hyperphosphorylation of the protein and an increased expression of the protein. Calreticulin must be cytosolic in order for it to function as a modulator of viral replication. Zhu and Newkirk (1994) observed that viral infection of human fibroblasts (MRC-5) with cytomegalovirus (CMV) led to cell surface expression of calreticulin and was postulated to provide a target for circulating autoantibodies and contribute to the autoimmune process. It has been suggested that viruses may be an etiological factor for autoimmune diseases, causing immunosuppression and thus may lead to the potential calreticulin-associated autoimmune diseases such as LEC (Yokoi *et al.*, 1993), rheumatoid arthritis, SLE or Sjorgen's Syndrome (Einsele *et al.*, 1992; Vasquez *et al.*, 1992; and Thorn *et al.*, 1988). After viral infection, calreticulin, normally in the lumen of the ER, may find its way to the cytoplasm or cell surface and thus be relocated and possibly modified (as in the case of rubella viral infection) and may now become immunogenic in the appropriate environment. Further experiments will have to be carried out to firmly investigate how calreticulin changes its location upon viral infection and hence contributes to the disease process.

Connected with another disease process, a calreticulin-like protein (Sm58) has been found in the homogenates of cercariae and in adult worms of *Schistosoma mansoni* (Khalife *et al.*, 1994). *Schistosoma mansoni* is the causative agent of schistosomiasis, a tropical parasitic disease estimated to infect 200 - 300 million people world wide. The life cycle of *Schistosoma mansoni* includes infection by cercariae which penetrates the host's skin, transforming into schistosomula, entering into the venous system of its host, migrating through the lungs, and then residing in the mesenteric veins as adults (Hawn *et al.*, 1993). Sm58 was expressed in the epithelia of the digestive duct and in the genital organs of the cercariae and the schistosomula and expression of the protein was found to be higher in adult worms (Khalife *et al.*, 1994). The presence of calreticulin in these glands suggested that the protein may be secreted/excreted from the cercariae, most probably during the invasive phase of the life cycle of *Schistosoma mansoni*. Ca^{2+} appears to be a vital physiological requirement for muscle contraction and penetration of the skin by cercariae (Davies, 1983). The results suggest that Sm58 (calreticulin), by regulating the Ca^{2+} concentration in schistosomes, may play an important role during cell proliferation (Khalife *et al.*, 1994) and hence contribute directly to the disease process of *Schistosoma mansoni*. Calreticulin may also be regulating the Fe^{3+} flow within the digestive tract of its host and thus contributing to the entry of the cercariae.

Calreticulin's NH_2-terminal amino acid sequence has also been found in the B50 melanoma antigen and in the complement 1 receptor molecule (C1qR). B50 was originally identified as a melanoma specific antigen (Gersten *et al.*, 1991), but has now been found on the cell surface of 29 cell lines tested (Gersten *et al.*, 1991). The C1qR has also been detected on a wide range of cells including peripheral blood B cells, T cells, monocytes, platelets, tissue macrophages, fibroblasts, and endothelial cells (Peerschke *et al.*, 1993; Malhotra *et al.*, 1993). In all the cells tested by Opas *et al.* (1991), no cell surface staining for calreticulin was observed.

Two very unusual observations relate to a possible secretion or membrane association of calreticulin. Hossain *et al.* (1991) have identified a Joro spider toxin (JSTX) specific binding protein in the membraneous fraction of bovine brain that had a similar NH_2-terminal amino acid sequence to calreticulin. The presence of a calreticulin-like protein in the secretions of the Ixodid tick salivary glands during feeding has also been described (Jaworski *et al.*, 1995). Joro spider toxin is isolated from the venom of the spider *Nephila clavata* and has been recognized as a potent glutamate blocker (Kawai *et al.*, 1982). Hossain *et al.* studied the effect of JSTX on Ca^{2+} elevation in cultured cells stimulated with glutamate and found that JSTX bound to a specific 60 kDa protein that was later found to have an identical NH_2-terminal amino acid sequence as calreticulin. However, the JSTX binding protein had a different amino acid composition from calreticulin, especially in the content of glutamic acid, glycine and valine. A complete amino acid sequence of this related protein will enable researchers to define its relationship to calreticulin's as possible role as a JSTX binding protein.

The presence of calreticulin in the secretions of Ixodid tick represents the most diverse aspect of calreticulin research to date. Ixodid ticks feed for days to several weeks and salivary secretions produced during this lengthy parasite-host relationship contain a variety of components that is thought to modulate events at the attachment site

(Kaufman, 1989). Secreted salivary factors include anti-complement (Ribeiro, 1987), anticoagulants (Neeper *et al.*, 1990), and anti-neutrophil compounds (Ribeiro *et al.*, 1990). Jaworski *et al.* provided evidence that a female ixodid tick's salivary glands contained and secreted a calreticulin-like protein. The function(s) of the calreticulin-like protein in salivary gland physiology or the host-tick interaction is unknown and requires greater investigation. Kuwabara *et al.* (1993) previously showed that calreticulin may have a potent anticoagulant activity in the canine model used in their study. We could postulate that the ixodid tick utilizes this function of calreticulin to prevent the closure of the attachment site in order to properly feed on its host. The completed cDNA sequence reported by Jaworski *et al.* (1995) revealed that the tick form of calreticulin lacked the KDEL ER retention sequence but contained the HEEL sequence. This weaker retention signal may explain why it is secreted and appearing in the salivary secretions of the Ixodid tick. The sequence did reveal a very homologous P-domain region, and an acidic carboxyl-terminus to calreticulin. A more detailed analysis of the sequence and characteristics of this calreticulin-like protein will allow for a better understanding of the similarities and differences between calreticulin and this calreticulin-like protein in order to understand the physiological role(s) calreticulin may have in the tick salivary gland.

HYPOTHESIS

Calreticulin is a Ca^{2+} binding protein found primarily in the lumen of the endo(sarco)plasmic reticulum (ER/SR), in the nuclear envelope and in the nucleus. Preliminary information concerning the amino acid sequence of calreticulin were obtained form the isolated cDNAs encoding the rabbit and mouse proteins by Fliegel *et al.* (1989b) and Smith and Koch (1989), respectively. Based on the amino acid sequence of calreticulin, both groups proposed that the protein can be divided into three distinct domains. The hypothesis for this work was that calreticulin may perform different functions that may be localized to different domains within the protein. The major goal of my research was an analysis of the structure and possible function of the different domains of calreticulin. The specific research objectives of this thesis were to:

i) develop a procedure for the purification of native calreticulin, recombinant calreticulin, and the different domains of protein (expressed as recombinant fusion proteins);

ii) analyze and identify the Ca^{2+} binding sites within the native, and recombinant protein as well as within calreticulin domains;

iii) localize and characterize the high affinity/low capacity Ca^{2+} binding site of calreticulin;

iv) analyze and identify the Zn^{2+} binding sites within calreticulin and calreticulin domains;

v) determine the influence of other ions, such as Mg^{2+}, on both Ca^{2+} and Zn^{2+} binding sites within calreticulin;

vi) determine what proteins may bind to calreticulin and how it may influence the ion binding abilities of calreticulin.

The proposed work will provide important, new information about the structure of calreticulin's high affinity, low capacity non-EF hand type of Ca^{2+} binding site, the mode of its interaction with cellular cofactors, as well as the role of these interactions in membrane structure and function. Such findings may suggest interpretations of clinical features in diseases involving abnormalities in related proteins, such as the Ro/SS-A autoantigen and other disease manifestations.

CHAPTER TWO

Experimental Procedures

A version of this chapter has been published - Baksh S. and M. Michalak. 1991. *J. Biol. Chem.* 266: 21,458 - 21,465; Baksh S. *et al.* 1992. *Protein Express. and Purification* 3: 322 - 331.; Baksh, S. *et al.* 1995. Zn^{2+} Binding to Calreticulin and Interaction with Protein Disulfide Isomerase. *J. Biol. Chem.* (submitted).

Materials

Imidazole, Tris, HEPES, DTNB, and Triton X-100 were purchased from Sigma. MOPS was from Fisher; Stains-All from Bio-Rad; Tween-20 and DEPC were from BDH. $^{45}CaCl_2$, ^{32}P-γATP, and $^{65}ZnCl_2$ were obtained from New England Nuclear. Peroxidase-conjugated rabbit anti-goat IgGs and peroxidase-conjugated goat anti-rabbit IgGs were from Bio-Rad and Boehringer Mannheim, respectively. Peroxidase-conjugated rabbit anti-GST IgGs were from Molecular Probes, Inc. Nitrocellulose and Immobilon (polyvinylidene difluoride) membrane filters were from Schleicher and Schuell and Millipore, respectively. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents and molecular weight markers were from Bio-Rad and Mandel Scientific. Endoglycosidase F, D and H were from Boehringer Mannheim; glycopeptidase F and Protein Kinase C were from Sigma Chemicals. Dimethylsulfoxide, benzamidine, phenylmethylsulfonyl fluoride, (N-[N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine, aprotinin, leupeptin, pepstatin, L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride, L-1-chloro-3-(4-tosylamido)-4-phenyl-2-butanone, (4-amidinophenyl)-methansulfonyl flouride, and phosphoramidone were obtained from Boehringer Mannheim. Reagents for SACH synthesis - salicylaldehyde and carbohydrazide were obtained from Sigma Chemicals. Taq polymerase was purified by the procedure of Engelke *et al.* (1990). Mono Q 5/5 FPLC, Resource Q, Superose 12/12 columns were from Pharmacia. DEAE-Sephadex A-50, Hydroxylapaptite beads, CM Sephadex C-50, and activated CNBr Sepharose beads were obtained from Pharmacia Biotechnologies. Oligodeoxynucloetides were synthesized in the DNA

Facility Lab of the Department of Biochemistry, University of Alberta. Peptides for antibody production and functional studies were synthesized by the PENCE group, University of Alberta. Fresh bovine liver was obtained from a local slaughterhouse and fresh porcine uterus was a generous gift of Dr. Y. Coe (Department of Pediatrics, University of Alberta). Canine pancreas was obtained from the Surgical Medical Research Institute, University of Alberta. All chemicals were of the highest grade available.

Methods

Purification of Native Calreticulin

Native calreticulin was isolated from fresh canine pancreas by a selective ammonium sulfate precipitation procedure. This method involved direct extraction of calreticulin from whole tissue homogenates and eliminated the need to prepare any membrane fractions. It was developed originally in Dr. Campbell's laboratory as a procedure for the isolation of muscle calsequestrin (Slupsky *et al.*, 1987) and was adopted by us for the purification of calreticulin. The purification of calreticulin was carried out in the presence of a mixture of protease inhibitors. The protease inhibitors used were first dissolved as a 2,000 x concentrated stock solution in 100% ethanol and used at the following final concentrations: 0.01 mg/ml of aprotinin, phosphoramidone, L-1-Chloro-3-(4-tosylamido)-7-amino-2-heptanone hydrochloride), L-Chloro-3-(4-tosylamido)-4-phenyl-2-butanone), (4-Amidinophenyl)-methanesulfonyl fluoride, N-[N-(L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl]-agmatine), 0.05 mg/ml leupeptin and 0.01 mg/ml pepstatin. This mixture was used in the preparation of calsequestrin, PDI and the fusion proteins and will be referred to throughout the thesis as "protease inhibitors" and was always used at 0.5 ml protease inhibitors per 100 ml of protein solution. Benzamidine hydrochloride (0.5 mM final concentration) and PMSF (0.5 mM final concentration) were made as 200 x concentrated stock solutions in 100% ethanol. All procedures were carried out at 4 ^{o}C unless otherwise stated. The tissue was homogenized at high speed in a Waring Blendor for 1 min, in 4 ml/g tissue of 100 mM KH_2PO_4, pH 7.1, containing 2.66 M ammonium sulfate (55% saturation), 1 mM EDTA and a mixture of the protease inhibitors as listed above. The homogenate was spun at 12 000g (9 000 rpm) for 30 min using a Sorvall GSA rotor. The supernatant was filtered through cheesecloth and saved. The pellet was rehomogenized in the same buffer for 1 min using half the original volume. The homogenate was centrifuged as before, and the supernatant was filtered and combined with the first supernatant: 200 g of solid ammonium sulfate was added per liter of supernatant to achieve 85% saturation of ammonium sulfate, and the suspension was stirred for 30 min at 4 ^{o}C. The pH was adjusted to 4.7 using phosphoric acid, the suspension was stirred for 30 minutes at 4 ^{o}C and centrifuged at 12 000g (9 000 rpm) for 30 min using a Sorvall GSA rotor. The pellet was dissolved in 100 mM KH_2PO_4, pH 7.1, containing 1 mM EGTA, and dialyzed for 15 - 25 h against the same buffer containing 50 mM NaCl. The solution was clarified by centrifugation at 12 000g (9 000 rpm) for 10 min. The resulting supernatant was applied directly to a DEAE-Sephadex A-50 column (3 cm x 12 cm; 60 ml bed volume; flow rate 0.6 ml/min; capacity 200 mg) equilibrated with 100 mM KH_2PO_4, pH 7.1, containing 1 mM EGTA and 50 mM NaCl. The column was washed with 5 volumes of the initial buffer, and then a linear gradient of NaCl from 50 mM to 750 mM was applied. Five ml fractions were collected, and the protein content of each fraction was monitored by the absorption at 280 nm, by the Lowry protein assay, by SDS-PAGE and by reactivity with anti-calreticulin antibodies. Calreticulin eluted between 300 - 400 mM NaCl. Fractions containing calreticulin were pooled and dialyzed for 16 hours against 10 mM K_2HPO_4, pH 7.0. Further purification of calreticulin was achieved by hydroxylapatite chromatography as described by MacLennan (1974). The sample was loaded onto a freshly packed hydroxylapatite column (2 cm x 15 cm; 50 ml bed volume; flow rate 0.5 ml/min; capacity 20 mg) and unbound protein was washed out with 150 ml of 10 mM K_2HPO_4, pH 7.0. The bound proteins were eluted with a 400 ml linear gradient of 10 mM to 1000 mM K_2HPO_4, pH 7.0. Five ml fractions were collected and analyzed for the presence of calreticulin by SDS-PAGE and reactivity with anti-calreticulin antibodies. Calreticulin eluted at approximately 213 mM K_2HPO_4. The peak fractions containing calreticulin were pooled, dialyzed against 10 mM ammonium bicarbonate, freeze-dried and stored at - 20 ^oC until use. I have used this procedure to isolate calreticulin from the following tissues: rabbit liver, skeletal muscle, cardiac muscle, kidney, pancreas and uterus, canine pancreas, human liver and pancreas, bovine liver, pancreas and uterus.

Purification of calsequestrin

Calsequestrin was isolated from the canine heart by the selective ammonium sulfate precipitation procedure as described above for calreticulin. This method also involved direct extraction of calsequestrin from whole tissue homogenates and eliminated the need to prepare any membrane fractions. All procedures were carried out at 4 ^{o}C. In the pre-DEAE-Sephadex stages, the calsequestrin preparation differed from the calreticulin preparation in the following step: 150 g of solid ammonium sulfate was used per liter of supernatant to achieve 85% ammonium sulfate. After application to the DEAE-Sephadex column, calsequestrin eluted off with a higher salt concentration than calreticulin at ~ 650 mM NaCl. Fractions from the DEAE-Sephadex column enriched in calsequestrin were further purified by Phenyl-Sepharose chromatography, as described by Cala and Jones (1983). The DEAE-Sephadex calsequestrin peak was dialyzed against buffer A (10 mM MOPS, 0.5 mM EGTA, 0.5 M NaCl, 1mM DTT, and 0.02% NaN_3, pH 7.0), and then mixed with phenyl-Sepharose beads overnight at 4^{o}C. The beads were then loaded onto a glass column (2 cm x 7 cm; 20 ml bed volume; flow rate 0.5 ml/min; capacity 30 mg), and then washed with 3 column volumes of buffer A. Calsequestrin was then eluted with 2 column volumes of 10 mM $CaCl_2$ in buffer A. Remaining proteins were eluted with 2 column volumes of low ionic strength buffer containing 10 mM MOPS, 1 mM DTT, and 0.02% NaN_3, pH 7.0. The column was regenerated by washing with 3 column volumes of buffer A. Calsequestrin was eluted from the Phenyl-Sepharose column in the presence of Ca^{2+}. The Ca^{2+} dependent elution of calsequestrin on the Phenyl-Sepharose column is based on the decrease in calsequestrin's surface hydrophobicity upon Ca^{2+} binding, resulting in a weakend interaction with the hydrophobic Phenyl-Sepharose matrix, enabling it to be eluted from the column.

Purification of protein disulfide isomerase (PDI)

PDI was purified from bovine liver as described by Lambert and Freedman (1983) with minor modifications. Frozen bovine liver was thawed overnight at 4 °C and homogenized at in 1 L of a buffer containing 100 mM sodium phosphate, pH 7.5, 5 mM EDTA, 1% v/v Triton X-100, 0.5 ml protease inhibitors/100 ml buffer, 0.5 mM PMSF and 0.5 mM benzamidine. Homogenization was carried out using a Waring Blendor at full speed for 4 X 30 sec bursts. The homogenate was strained through two layers of muslim, centrifuged at 18 000g (13 000 rpm) for 30 mins using a Sorvall GSA rotor and the pellet discarded. The supernatant was placed in a water bath at 70 °C with constant stirring and the temperature of the extract was allowed to reach 54 °C ± 1 °C , where it was maintained for 15 min, cooled on ice and then centrifuged at 18 000g (13 000 rpm) for 40 mins as before. The pellet was discarded and to the supernatant 351 g of solid ammonium sulfate was added per liter to achieve 55 % saturation of ammonium sulfate. After stirring at 4 °C for 30 mins, the precipitated supernatant was centrifuged at 18 000g (13 000 rpm) for 30 mins using a Sorvall GSA rotor. The pellet was discarded and to the resultant supernatant 218 g of solid ammonium sulfate was added per liter to achieve 85 % saturation of ammonium sulfate, stirred at 4 °C for 30 mins, and then centrifuged as before. The supernatant was discarded and the pellet was resuspended in 20 mls of 25 mM sodium citrate, pH 5.3 and dialyzed overnight against the same buffer. The sample was clarified by centrifugation at 12 000g (9 000 rpm) for 12 minutes using a JA-17 rotor. Further purification of PDI involved a carboxylmethylcellulose (CM) column instead of the carboxylmethyl-Sephadex column described by Lambert and Freedman (1983). This was the only available matrix at the time and it utilized the same cation exchange properties as the carboxylmethyl-Sephadex matrix used by Lambert and Freedman (1983). The sample was loaded onto the carboxylmethylcellulose cation exchange column (2 x 15 cm/50 ml bed volume; flow rate. 0.4 ml/min; capacity, 20 mg) previously equilibrated with the 25 mM citrate, pH 5.3 and then eluted with the same buffer at a flow rate of 3-4 ml/min. Eluted proteins (containing PDI and other acidic proteins) were collected and analyzed on SDS-PAGE. Peaks containing proteins immunoreactive with the anti-PDI antibody were pooled, concentrated, and solid ammonium sulfate added to achieve 100 % saturation. The solution was stirred at 4 °C for 30 min, and then centrifuged at 18 000g (13 000 rpm) for 40 min using a Sorvall GSA rotor. The amount of ammonium sulfate required to achieve 100% saturation was calculated by using the relationship - g ammonium sulfate required for 100% saturation = 0.761 g X mls of solution. The precipitated sample was then centrifuged at 18 000g (13 000 rpm) for 30 min., the supernatant discarded and the pellet resuspended in 15 mls of 20 mM sodium phosphate, pH 6.3 and dialyzed extensively against this buffer (2 X 4 L). Following dialysis the sample was clarified by centrifugation at 12 000g (9,000 rpm) for 12 minutes using a JA-17 rotor and then applied to a Mono Q cation FPLC column, previously equilibrated with 20 mM sodium phosphate, pH 6.3 (column buffer). Proteins were eluted with a linear salt gradient from 0 to 700 mM NaCl (0.33 mM/min) in the column buffer and 2.5 ml fractions were collected. Three major protein peaks were detected by absorbance at 280 nm. The middle peak which eluted at ~350 mM NaCl contained PDI as confirmed by its mobility on SDS-PAGE and by its immunoreaction with antibodies against PDI. PDI immunoreactive fractions were pooled, concentrated and dialyzed against 10 mM Tris, pH 7.0, freeze-dried and stored at - 20 °C until use.

PDI Assay

Activity of PDI was followed by PDI's ability to refold scrambled RNase A which in turn will regain its ability to cleave highly polymerized yeast RNA (Sigma Chemicals, Inc.) (Lambert and Freedman, 1983). For this assay, PDI was dissolved to 2.0 µg/ml in 100 mM sodium phosphate pH 7.5, 2 mM glutathione, 0.2 mM reduced glutathione. The reaction was started by adding scrambled RNase A (obtained using the procedure of Hillson *et al.*, 1984) (200 µg/ml) into the reaction mixture. Ten µl aliquots were taken at various times and placed in 3 ml of a solution containing 50 mM Tris, pH 7.0, 2.5 mM KCl, 5 mM $MgCl_2$ followed by incubation for 2 min. at 25°C. The A_{260}/A_{280} ratio was measured as indicative of the extent of cleavage of yeast RNA by re-folded RNase A. Fully folded RNase A (Sigma Chemicals, Inc.) was used as a control. This experiment was repeated in the presence of 1 : 1 ratio of calsequestrin or GST (used a controls), calreticulin or calreticulin domains in order to determine the what effect these proteins have on the ability of PDI to refold scrambled RNase A.

Preparation of Antibodies and Immunoblotting

Calreticulin and PDI were purified by ammonium sulfate precipitation as previously described and were used in the production of specific antibodies. Rabbits and goat were immunized with 0.1 mg and 1 mg of the appropriate protein emulsified in Freund's complete adjuvent, respectively. After 2 weeks and 4 weeks, the immunization was repeated using Freund's incomplete adjutant. Antibodies were screened by immunoblotting proteins after electrophoretic transfer onto a nitrocellulose membrane, according to the method of Towbin *et al.* (1979).

Goat anti-rabbit calreticulin was found to recognize only the P-domain and the C-domain of calreticulin (see Fig. 3-5C). This antibody was used for the purification of a pool of antibodies that would selectively recognize only the P-domain of calreticulin. A GST-P-domain fusion protein affinity column was synthesized by coupling the purified GST-P-domain fusion protein to CNBr activated Sepharose 4B (Pharmacia) by the protocol recommended by the manufacturer. Two mg of GST-P-domain was coupled to 1 ml of activated CNBr-Sepharose 4B by rotation in coupling buffer (100 mM $NaHCO_3$, pH 8.3 and 500 mM NaCl) for 16-18 hours at 4 °C. Unbound GST-P-domain was removed by sequential washes in coupling buffer. Remaining active groups were then blocked for 1 hour at room temperature by incubation in a solution containing 1 M ethanolamine and 0.1 M $NaHCO_3$, pH 9.0. The column was further washed three times with a low pH buffer (100 mM sodium acetate, 500 mM NaCl, pH 4.0) followed by washing with a high pH buffer (100 mM Tris and 500 mM NaCl, pH 8.0). This washing step removed any excess absorbed proteins. A measurement of the protein concentration before and after coupling, revealed a coupling efficiency of greater than 95%. The beads were brought to room temperature and washed with 2 - 4 bed volumes of Immunopure elution buffer (Pierce). Before application of the antibody sera to be affinity purified, the beads were equilibrated with 5 bed volumes of the application buffer (TBS pH 6.8). The goat anti-rabbit calreticulin antibody was added to the beads and the sample was allowed to rotate end-over-end at 4 ^{o}C for 16 hours. The mixture was poured into a 1 ml glass column, washed with 2 bed volumes of TBS containing 500 mM NaCl and the flowthrough was collected. The column was washed with 2 bed volumes of TBS, and bound antibodies were eluted with 5 bed volumes of Immunopure elution buffer (Pierce). Bovine serum albumin was added at 1 mg/ml to stabilize the affinity purified antibody solution and affinity purified antibodies were then stored at -20 ^{o}C. The column was regenerated by washing with several volumes of TBS.

Immunobloting was carried out as described by Michalak *et al.* (1990). Briefly, blocking of immunoblot was carried out by incubating in 5% milk powder in PBS (0.137 M NaCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4). This was followed by incubation with the appropriate primary antibody at 1:200 - 1:300 dilution in PBS and 1% milk powder. After 1.5 hours, the blot was washed two times for five minutes in PBS containing 0.05% Tween-20, followed by one five minute incubation in PBS. The blot was incubated with the appropriate peroxidase-conjugated secondary antibody at 1:2000 dilution in PBS and 1% milk powder. After another 1.5 hours the blot was washed twice in PBS containing 0.05% Tween-20, followed by one wash in PBS (as before). Antibody binding was detected using the ECL reagents (Amersham) which are activated by the peroxidase groups on the peroxidase-conjugated secondary antibodies. These groups activate the ECL reagents to release light energy that is picked up by the X-ray film and then visualized by autoradiographed.

Glutathione fusion protein system

The Glutathione S-transferase (GST) fusion system (Pharmacia) was used for the expression and isolation of recombinant calreticulin and recombinant calreticulin domains. This system utilizes the IPTG-inducible prokaryotic expression vector pGEX-3X (Smith and Johnson, 1988) . pGEX-3X codes for GST followed by a Factor Xa cleavage site (I-E-G-R), a multiple cloning site and a stop codon (Fig. 2-1). The Factor Xa cleavage site allows for the proteolysis of fusion proteins in order to obtain calreticulin domains that are free of GST.

Construction of mature calreticulin and calreticulin domains

cDNAs encoding the mature form of calreticulin or various regions of the protein (the N-, P-, C-domains) were inserted into pGEX-3X to form the following plasmids: pGEX-CRT, pGEX-N, pGEX-P, and pGEX-C, respectively (Figs. 2-2 to 2-3). pcDx-CRT containing the cDNA encoding rabbit skeletal muscle calreticulin (Fliegel *et al.*, 1989b) was used for construction of these vectors (see Fig. 2-2a). cDNA fragments encoding the mature full length form of calreticulin (i.e. minus a 17 amino acid leader peptide) (Fig. 2-2a) or for the N-domain (amino acid #1-200) (Fig. 2-2b), or the N+P-domain (amino acid #1-273) (Fig. 2-3a) were PCR amplified using Sal I linearized pcDx-CRT as a template. The nucleotide

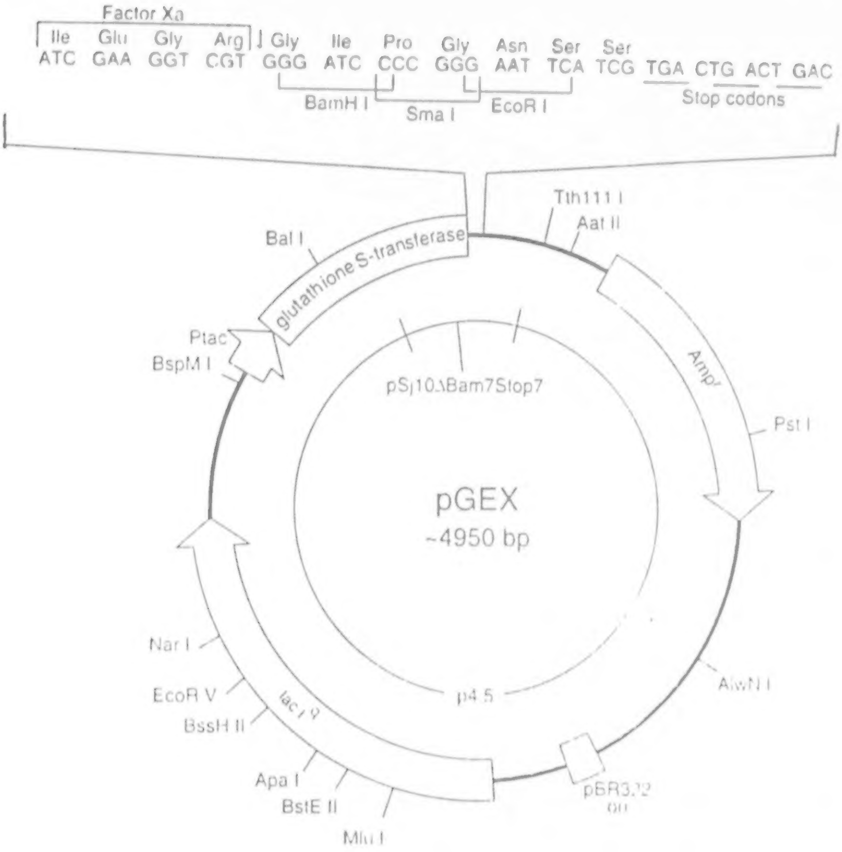

Factor Xa
Ile Glu Gly Arg
ATC GAA GGT CGT
Gly Ile Pro Gly Asn Ser Ser
GGG ATC CCC GGG AAT TCA TCG TGA CTG ACT GAC
BamH I
Sma I
EcoR I
Stop codons
Tth111 I
Aat II
Bal I
glutathione S-transferase
Ptac
BspM I
pSj10ΔBam7Stop7
Amp^r
Pst I
pGEX
~4950 bp
Nar I
EcoR V
BssH II
lac I^q
p4.5
AlwN I
Apa I
BstE II
Mlu I
pBR322 ori

Figure 2-1: Plasmid map of pGEX-3X vector

This vector was used in the Glutathione S-transferase fusion protein system. This system utilizes the prokayotic expression vector pGEX-3X described by Smith and Johnson (1988). It codes for GST followed by the Factor Xa cleavage site (I-E-G-R), a multiple cloning site (Bam H1/Sma 1/ Eco R1), and a stop codon.. Adapted from Pharmacia Biotechnologies.

Figure 2-2: Plasmid map of (a) pGEX-CRT and (b) pGEX-N

cDNA encoding full length mature calreticulin (a) and N-domain (b) were amplified by PCR using Sal 1-linearized pCDx-CRT (Fliegel *et al.*, 1989b) as a template. The PCR products were subcloned into the Eco R1 site of the pGEX-3X plasmid. The nucleotide sequences of pGEX-CRT and pGEX-N were confirmed by DNA sequencing. Modified, with permission, from Kimberly Burns, Ph. D. dissertation (1994).

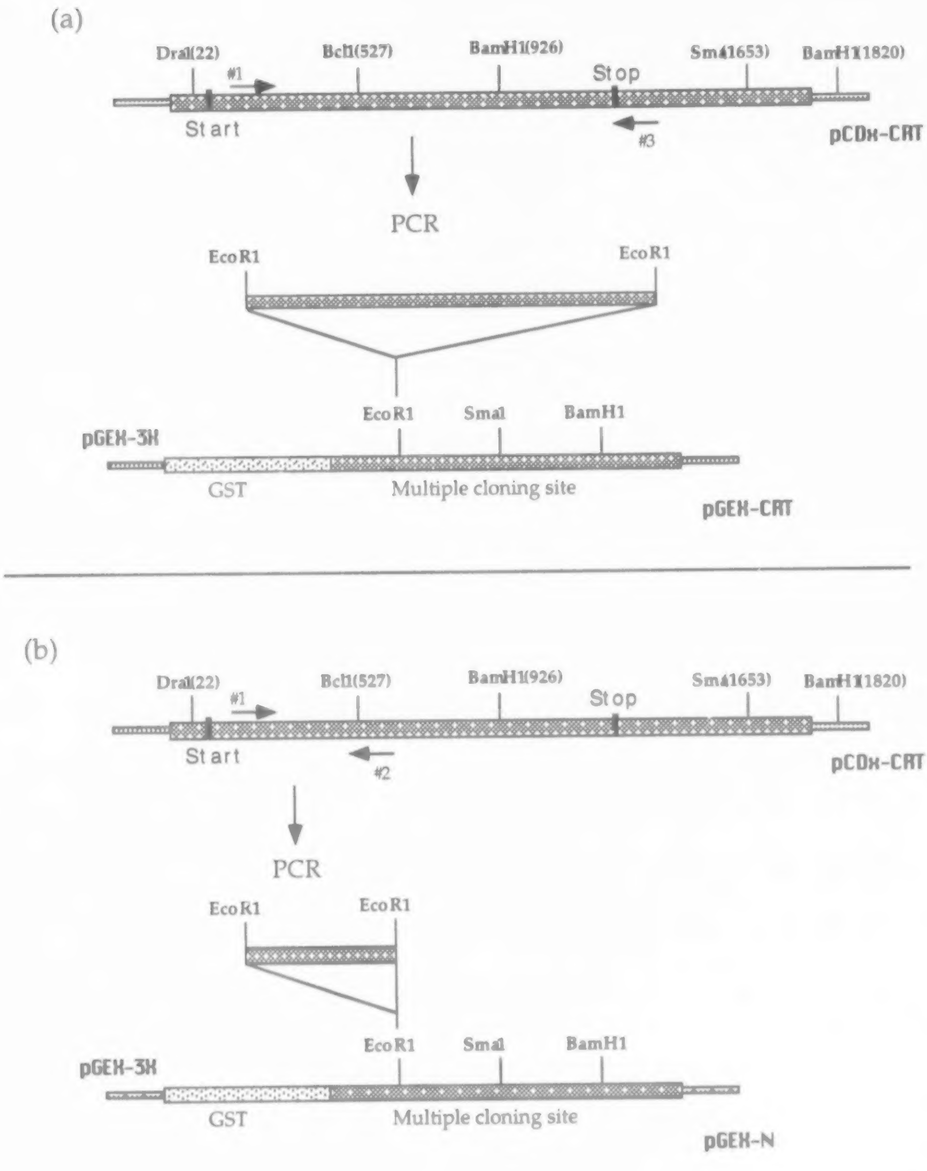
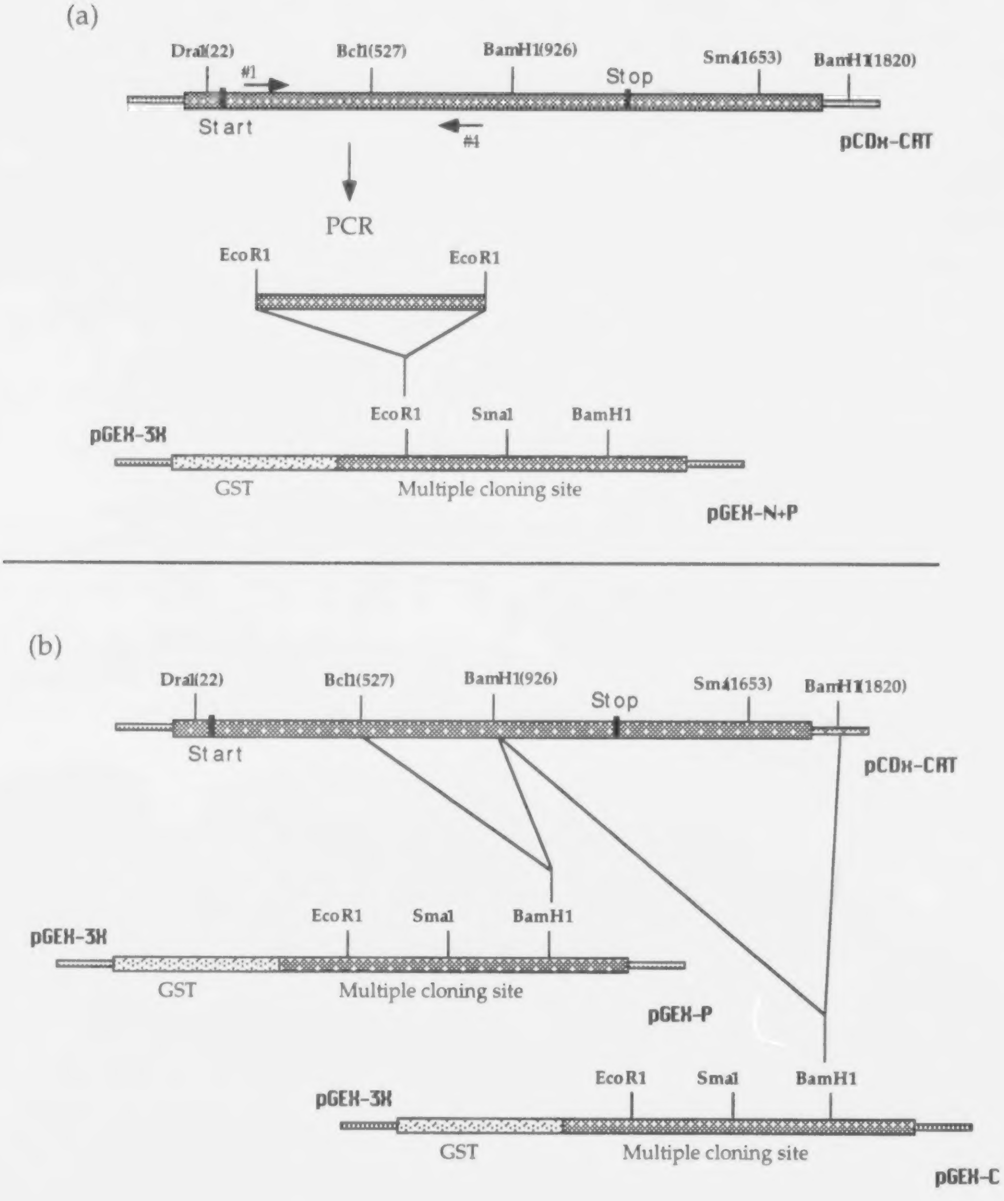

(a)
DraI(22)
BclI(527)
BamH1(926)
SmaI(1653)
BamH1(1820)
#1
Stop
Start
#3
pCDx-CRT
PCR
EcoR1
EcoR1
EcoR1
SmaI
BamH1
pGEX-3X
GST
Multiple cloning site
pGEX-CRT
(b)
DraI(22)
BclI(527)
BamH1(926)
SmaI(1653)
BamH1(1820)
#1
Stop
Start
#2
pCDx-CRT
PCR
EcoR1
EcoR1
EcoR1
SmaI
BamH1
pGEX-3X
GST
Multiple cloning site
pGEX-N

Figure 2-3: (a) Plasmid map of pGEX-N+P; (a) pGEX-P and pGEX-C

(a) cDNA encoding the N+P-domain was amplified by PCR using Sal 1-linearized pCDx-CRT (Fliegel *et al.*, 1989b) as a template. The PCR product was subcloned into the Eco R1 of the pGEX-3X plasmid. The nucleotide sequence of pGEX-N+P was confirmed by DNA sequencing.

(b) cDNA encoding the P-domain and C-domain were obtained by digestion of pCDx-CRT (Fliegel *et al.*, 1989b) with Bcl 1/Bam H1 and Bam H1/Bam H1 respectively. The resulting restriction fragments were ligated into the Bam H1 site of the pGEX-3X plasmid. Modified, with permission, from Kimberly Burns, Ph. D. dissertation (1994).


(a)
Dra1(22)
Bcl1(527)
BamH1(926)
Stop
Sma1(1653)
BamH1(1820)
#1
Start
#4
pCDx-CAT
PCR
EcoR1
EcoR1
pGEX-3X
EcoR1
Sma1
BamH1
GST
Multiple cloning site
pGEX-N+P
(b)
Dra1(22)
Bcl1(527)
BamH1(926)
Stop
Sma1(1653)
BamH1(1820)
Start
pCDx-CAT
pGEX-3X
EcoR1
Sma1
BamH1
GST
Multiple cloning site
pGEX-P
pGEX-3X
EcoR1
Sma1
BamH1
GST
Multiple cloning site
pGEX-C

sequences of the DNA primers used to generate these cDNA fragments is shown in Table 2-1 and the PCR reactions are outlined in Table 2-2. PCR reactions were carried out in a buffer containing 50 mM KCl, 10 mM Tris, pH 8.3, 0.1% gelatin, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 2.5 units of TaqI polymerase and 100 pmole of the appropriate primer. The PCR reactions were carried out for 25 cycles consisting of 1 min. at 94 °C, 1 min. at 57 °C (mature calreticulin) or 52 °C (N-domain and N+P-domain) and 2 min. at 72 °C. Amplification was completed by a final incubation at 72 °C for 10 min. PCR products were purified by polyacrylamide gel electrophoresis, digested with EcoR1 restriction endonuclease and ligated into an EcoR1 restriction site of the calf-intestinal phosphatase-treated pGEX-3X plasmid. The following plasmids were generated pGEX-CRT (Fig. 2-2a), pGEX-N (Fig. 2-2b) and pGEX-N+P (Fig. 2-3a) encoding full length mature calreticulin, N-domain, and N+P-domain of the protein, respectively. pGEX-CRT was sequenced to determine if there were any base changes as a result of the PCR. The insert encoding for full length mature calreticulin contained only five base changes, but only one change, at position #1286 (C-A), created an amino acid substitution from (alanine to glutamate) at residue #391. This change was considered to be a conservative one, since human calreticulin has an aspartate at this position; all the other plasmid sequences encoding calreticulin domains were correct.

Plasmids containing P-domain, C-domain and P+C-domain of the protein, designated pGEX-P (Fig. 2-3b), pGEX-C (Fig. 2-3b) and pGEX-P+C (Fig. 2-4a), respectively, were generated by subcloning of cDNA restriction fragments of the pcDX plasmid. Bcl1-BamH1 DNA fragment (nucleotide

Figure 2-4: (a) Plasmid map of pGEX-P+C; (b) primers for construction of truncated fragments of the P-domain of calreticulin

(a) cDNA encoding the P+C-domain was obtained by digestion of pCDx-CRT (Fliegel *et al.*, 1989b) with Bcl 1/Sma 1. The resulting restriction fragment was ligated into the Sma1/Bam H1 site of the pGEX-3X plasmid.

(b) The cDNA's for the truncated GST-P-domain fusion proteins were amplified by PCR using Sma 1-linearized pCDx-CRT (Fliegel *et al.*, 1989b) as a template. The PCR products were subcloned into the Eco R1 of the pGEX-3X plasmid. The nucleotide sequence of the inserts were confirmed by DNA sequencing.

Modified, with permission, from Kimberly Burns, Ph. D. dissertation (1994).

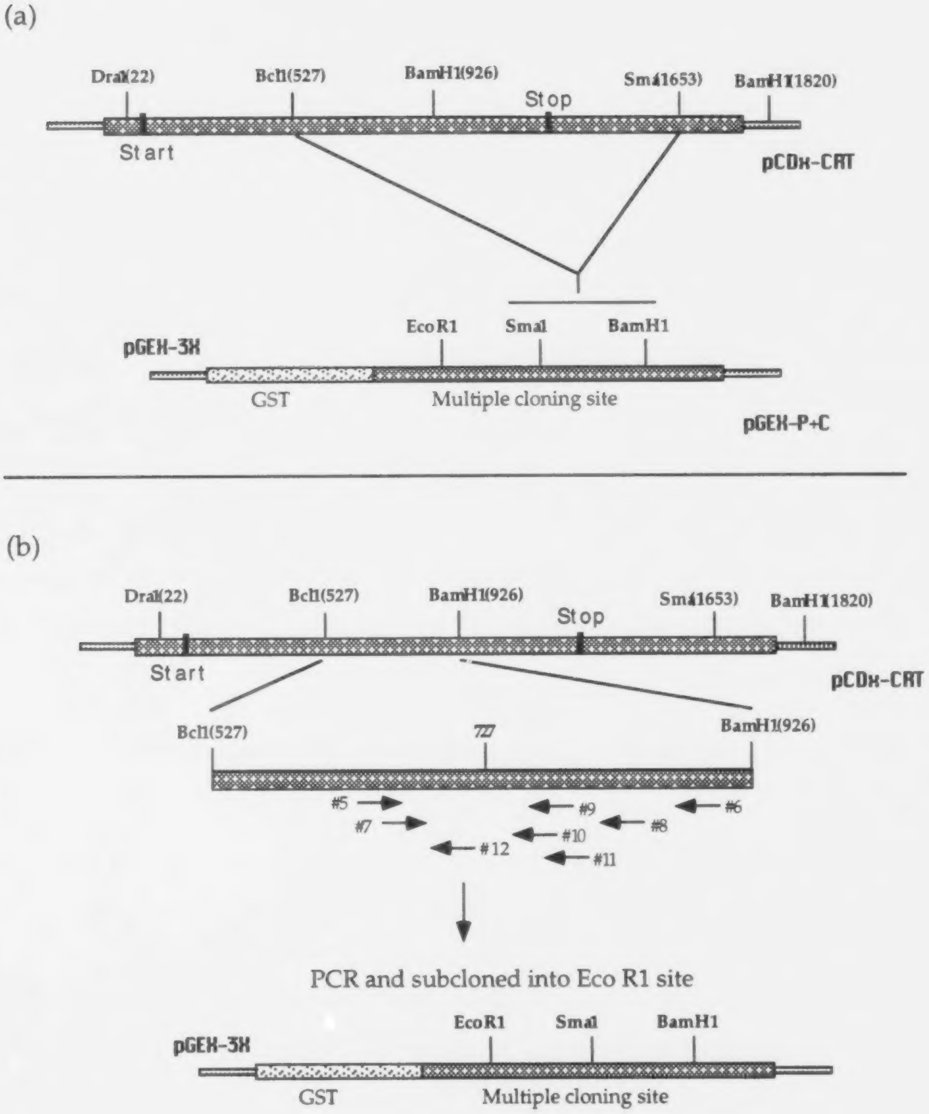

(a)
DraI(22)
BclI(527)
BamH1(926)
Stop
SmaI(1653)
BamH1(1820)
Start
pCDx-CRT
pGEX-3X
EcoR1
SmaI
BamH1
GST
Multiple cloning site
pGEX-P+C
(b)
DraI(22)
BclI(527)
BamH1(926)
Stop
SmaI(1653)
BamH1(1820)
Start
pCDx-CRT
BclI(527)
727
BamH1(926)
#5
#9
#6
#7
#10
#8
#12
#11
PCR and subcloned into Eco R1 site
pGEX-3X
EcoR1
SmaI
BamH1
GST
Multiple cloning site

Table 2-1: Primers for fusion protein constructs

Primer	Name	Sequence	Nucleotide Sequence #
#1	CRT	5′-atgaattcGGAGCCCGTCGTCTACTTCAA-3′	1 - 20
#2	N	5-′gggaattcACTACCAGTCATCCTCCAGGGA-3′	531 - 549
#3	CRT′	5′-gggaattcAGAGACATTATTTGGCTCTGCG-3′	1242 - 1264
#4	N+P	5′-tggaattcAGGCATAGATGTTAGCGTCGGG-3′	849 - 870
#5	MM18	5'-tggaattcGGAGGATGACTGGGACTTCCTA-3'	648 - 669
#6	SB-1	5′-tggaattcTAGATCCAGGTGCC-3′	919 - 930
#7	SB-2	5'-tggaattcGTTCCTACCCCCCAAGAAG-3'	664 - 681
#8	SB-3	5'-tggaattcCTACTGAATCACCGGCGGC-3'	846 - 861
#9	SB-4	5'-tggaattcCTACGCGTCCGGGTCGGGG-3'	786 - 801
#10	SB-10	5'-CTCGGGCTTGTCGAAGTCCTCGGGCTT-3'	754 - 780
#11	SB-7	5'-GTCCCAGTCTTCGGTCTTCTTCGC-3'	799 - 822
#12	SB-5	5'-GTCCCAGTCTTCAGTCTTCGAGGC-3'	696 - 720

Note: All primers have the Eco R1 restriction endonuclease site GAATTC (underlined) at their 5' position except for primers 10, 11, 12. Primer 3 encodes until 3 bp behind the stop codon of the Fliegel *et al.* (1989b) clone.

Table 2-2: PCR reactions for the construction of GST fusion proteins

Protein product	1st PCR Rxn (PCR1)		2nd PCR Rxn (PCR2)		Nucleotide Sequence	Amino Acid Sequence
	5' forward	**3' reverse**	**5' forward**	**3' reverse**		
GST-CRT	1	3	-----	-----	1 -1264	1 - 401
GST-N	1	2	-----	-----	1 - 549	1 - 182
GST-N+P	1	4	-----	-----	1 - 870	1 - 290
GST-HA1	5	6	-----	-----	648 - 930	180 - 273
GST-HA3	7	9	-----	-----	665 - 798	185 - 230
GST-HA5 (199P-->T) (204E-->K)	7	12	PCR1	8	665 - 861	185 - 250
GST-HA8 (233P --> 233T)	7	11	PCR1	8	665 - 861	185 - 245
GST-HA10(219W --> 219F)	7	10	PCR1	8	665 - 861	185 - 250 **

Note: GST-HA1, GST-HA10, GST-HA5 and GST-HA8 were expressed in DH5α E. coli; GST-CRT, GST-N, GST-N+P, and GST-HA3 were expressed in BNN103 E. coli cells. ** GST-HA10 is missing the first repeat sequence A (i.e., missing amino acids 191 - 214) (see Fig. 1-2B and Fig. 4-8A).

527 to 926) encoded the P-domain; BamH1-BamH1 fragment (nucleotides to 1820) encoded the C-domain; and Bcl1-Sma1 fragment (nucleotides 527 to 1653) encoded P+C-domain (Fliegel *et al.*, 1989b). The restriction fragments were separated by acrylamide gel electrophoresis, excised and glass milk purified, and subcloned into the BamH1 site (P- and C-domain) or BamH1/Sma1 site (P+C-domain) of the pGEX-3X. The newly generated plasmids were transformed into DH5α E. coli and/or into BNN103 E. coli.

Construction of fusion proteins corresponding to truncated fragments of the P-domain

In order to identify the high affinity Ca^{2+} binding site within the P-domain of calreticulin, truncated fragments of the P-domain were expressed as fusion proteins using the GST expression system. All constructs were PCR amplified using Sma1 linearized pcDx-CRT as a template. The synthetic oligodeoxynucleotides used to construct these short fragments within the P-domain are shown in Table 2-1 (primers 5 - 11) and Fig. 2-4b. PCR reactions were carried out in a buffer containing 50 mM KCl, 10 mM Tris, pH 8.3, 0.01% gelatin, 200 μM of each dNTP, 1.5 mM $MgCl_2$, 2.5 units of TaqI polymerase and 100 pmole of the appropriate primer and 60 ng of pCDx-CRT. The PCR reactions were carried out for 25 cycles consisting of a hot start (at 94 °C) for 2 min. followed by cycles consisting of 1 min. at 94 °C, 1 min. at 57 °C and 2 min. at 72 °C. Amplification was completed by a final incubation at 72 °C for 10 min. PCR products were separated by polyacrylamide gel electrophoresis, excised and glass milk purified, followed by digestion with EcoR1 restriction endonuclease. The resulting restriction fragment was subcloned into an EcoR1 restriction site of the phosphatase-treated pGEX-3X plasmid. Plasmids were then transformed into either DH5α or BNN103 E. coli cells. Ampicillin resistant positive clones were selected, the plasmid was isolated, and the DNA sequence was confirmed by reading off the primed sequence using a pGEX-3X 5' primer (located at nucleotide position 641 - 663 of the pGEX-3X plasmid):

5' - ACCATCCTCCAAAATCGGATCTG - 3'

This sequence is located at the carboxyl-terminal end of GST.

Expression and isolation of fusion proteins

All expression experiments were carried out using the BNN103 or DH5α as a host. BNN103 or DH5α transformed cells were grown overnight at 37 °C in 2 ml of LB containing 50 μg of ampicillin/ml. This was then transferred to 20 ml of LB containing 50 μg of ampicillin/ml, grown to mid-log phase (OD_{600} = 0.6 to 1.0), and the cells then induced with the addition of 0.1 mM IPTG and incubated for 4 hours at 37 °C. Cells were spun down at 3 500g for 20 min and the pellet was resuspended in PBS containing 0.1% Triton X-100 followed by lysis using a French Press set at 1,000 psi. The lysed extract was centrifuged at 10 000g (9 000 rpm using a JA-17 rotor) for 12 min and the supernatant (containing the soluble fusion protein) was then assayed for protein content, frozen and stored at - 20 °C until further use.

GST and GST-calreticulin fusion proteins were purified by one step Glutathione-Sepharose affinity chromatography (2 cm x 12 cm; 40 ml bed volume; flow rate 1 ml/min; capacity 7.5 mg/ml of beads). The E. coli supernatant was diluted with PBS, containing 1% Triton X-100, to a final protein concentration of 2 mg/ml and applied directly onto the Glutathione-Sepharose column equilibrated with the same buffer. The flow-through was reapplied onto the column and washed with 400 ml of PBS. The fusion proteins were eluted from the affinity column with 200 ml of a buffer containing 5 mM glutathione in 50 mM Tris-HCl, pH 8.0.

Factor Xa digestion was used to remove GST from the calreticulin fusion proteins. Factor Xa was prepared by the procedure described by Friedberg and Pizzo (1988). Proteolytic cleavage was carried out for 6 to 18 hours depending on the fusion protein used. Digestion was carried out in 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM $CaCl_2$ at room temperature and 1: 50 dilution of Factor Xa. The digestion was stopped by the addition of PMSF to a final concentration of 2.5 mM. GST was then removed from the sample by reapplication of the digested sample to a fresh Glutathione-Sepharose column. The column flowthrough (containing recombinant calreticulin or calreticulin domains) was collected and then further purified on a Mono Q FPLC cation column as described in "Chapter Three: Purification, expression, and biochemical characterization of calreticulin and calreticulin domains".

Molecular Cloning Methods

DNA fragments used in the construction of the different plasmids were isolated from agarose gel slices by glass milk elution (Davis *et al.*, 1986). Alkaline phosphatase treatment and other cloning procedures were carried out according to published protocols (Sambrook *et al.*, 1989; Ansubel *et al.*, 1992). Plasmid isolation was carried out as outlined by Morelle (1989). All ligations were carried out in ligase buffer at room temperature using a 3 : 1 ratio of plasmid to insert (Sambrook *et al.*, 1989). Following ligation for 16 hours, 1/3 to 1/2 of the ligation mixture was added to 100 μl of freshly thawed cells (DH5α or BNN103), incubated on ice for 10 min., heat pulsed at 42 °C for 90 sec., and placed on ice for 1 - 2 min. One ml of LB was added to the transformation mixture followed by the incubation at 37 °C for one hour. The cells were then spun down at 16 000g (14 000 rpm) for 1 min., resuspended in 100 μl of LB, and plated onto LB plates containing 100 μg/ml ampicillin. Positive clones were picked used for expression experiments as outlined above.

Calcium binding analysis

The free Ca^{2+} concentration was calculated on the basis of the EGTA association constant reported by Fabiato and Fabiato (1979). The calculation of free Ca^{2+} was accomplished by the use of a computer program that corrected for ionic strength, pH, EGTA and K^+.

$^{45}Ca^{2+}$ *overlay binding analysis.* $^{45}Ca^{2+}$ binding to proteins on nitrocellulose membranes was carried out by the method of Maruyama *et al.* (1984). Briefly, membranes were incubated and blocked for 4 hours in a solution containing 10 mM imidazole, pH 6.8, 5 mM $MgCl_2$, 60 mM KCl with changes every hour. The membrane was then incubated in the same buffer containing 1.23×10^{-6} M or 1.27×10^{-5} M free concentration of $^{45}Ca^{2+}$. $^{45}Ca^{2+}$ binding to proteins was visualized by autoradiography on Kodak X-OMAT AR films.

Equilibrium dialysis method The proteins were first dialyzed against 10 mM MOPS, pH 7.0, 150 mM KCl, and 0.1 mM EGTA. Protein aliquots (~300 - 600 µg) were dialyzed for 18 hours at 4 °C against 10 mM MOPS, 150 mM KCl, 0.1 mM EGTA, 0.64 µCi of $^{45}Ca^{2+}$/ml containing different concentrations of unlabeled Ca^{2+}. Molecules of $^{45}Ca^{2+}$ would enter by diffusion into the dialysis bag and some would be taken up by the Ca^{2+} binding protein. The remaining molecules of $^{45}Ca^{2+}$ would distribute itself between the outside and inside of the dialysis bag. This diffusion process would continue until an equilibrium is reached representing the maximal binding of $^{45}Ca^{2+}$ to the protein within the dialysis bag. The amount of $^{45}Ca^{2+}$ on the outside of the bag would represent the free concentration of $^{45}Ca^{2+}$ ($[^{45}Ca^{2+}]_{f\text{-}o}$) and the amount of radioactivity inside the dialysis bag represents a total $^{45}Ca^{2+}$ ($[^{45}Ca^{2+}]_t$). This is a total of free $^{45}Ca^{2+}$ inside of the bag ($[^{45}Ca^{2+}]_{f\text{-}i}$) and bound $^{45}Ca^{2+}$ to the Ca^{2+} binding protein ($[^{45}Ca^{2+}]_b$). The following relations are then true:

(1) $[^{45}Ca^{2+}]_{f\text{-}o} = [^{45}Ca^{2+}]_{f\text{-}i}$ if proper diffusion is achieved;
(2) $[^{45}Ca^{2+}]_t = [^{45}Ca^{2+}]_{f\text{-}i} + [^{45}Ca^{2+}]_b$
(3) Therefore, $[^{45}Ca^{2+}]_b = [^{45}Ca^{2+}]_t - [^{45}Ca^{2+}]_{f\text{-}i}$
(4) $[^{45}Ca^{2+}]_b = [^{45}Ca^{2+}]_t - [^{45}Ca^{2+}]_{f\text{-}o}$ because of (1)

The data for $[^{45}Ca^{2+}]_b$, $[^{45}Ca^{2+}]_t$, and $[^{45}Ca^{2+}]_{f\text{-}o}$ would be in counts per minute and can be converted to moles of Ca^{2+} by using the specific activity of the stock of $^{45}Ca^{2+}$ used in the experiment (28.80 mCi/ml; 17.30 mCi/mg). These numbers were divided by the amount of protein used to obtain the moles of Ca^{2+} bound per mole of protein (B in Scatchard plots). This number was then be divided by the amount of free Ca^{2+} and used in Scatchard plot analysis (B/F in plots). K_d and B_{max} were obtained from the plot of B/F versus B.

Ca^{2+} binding assays were carried out in triplicate using 2 or 3 different preparations of native calreticulin, recombinant calreticulin and calreticulin domains.

Ca^{2+} microelectrode method - Samples were prepared by dialyzing 1 mg of protein for 2 days against a buffer containing 10 mM MOPS, 150 mM KCl, 1 mM EDTA, pH 7.0 followed by a second dialysis for 2 days against a buffer containing 10 mM MOPS, 150 mM KCl, pH 7.0. This extensive dialysis protocol was used to remove endogenous divalent cations. The sample was then lyophilized and resuspended in 10 mM MOPS, 150 mM KCl, pH 7.0 when electrode measurements were done. This was done to concentrate the samples. An Orion Ca^{2+} microelectrode was used with a Ag/Ag^{2+} reference solution. A calibration curve was carried out using a 1/10 dilution of a standard 0.1 M solution of $CaCl_2$ purchased from Orion Laboratories. One microlitre Ca^{2+} additions were performed sequentially to obtain the range of Ca^{2+} concentrations from 10^{-6} M to 10^{-4} M. Millivolt readings were allowed to equilibrate for 2 min. and then recorded. The Ca^{2+} electrode readings were linear within this range and was sufficient to observe the high affinity Ca^{2+} binding to the fusion proteins. The Ca^{2+} binding assays were carried out in duplicate using 300 µg of fusion protein.

Millivolt readings in the absence (M_a) or presence (M_p) of the Ca^{2+} binding protein was carried out. M_a represents the amount of free Ca^{2+} and M_p represents the total amount of Ca^{2+} free in solution and bound Ca^{2+} to the Ca^{2+} binding protein. M_a can be used to plot a calibration curve of mV versus $[Ca^{2+}]$ and the amount of bound Ca^{2+} can be obtained by first reading off the calibration curve the $[Ca^{2+}]$ corresponding to the M_p millivolt readings and then subtracting this number from the $[Ca^{2+}]$ in the absence of protein. The amount of bound Ca^{2+} can then be divided by the amount of protein used to obtain the moles of Ca^{2+} bound per mole of protein (B value in Scatchard plots) and then divided by the amount of free Ca^{2+} to obtain the B/F value in Scatchard plots. K_d and B_{max} were obtained from the plot of B/F versus B.

Zn^{2+}binding analysis

Synthesis of Zn^{2+} sensitive dye - Salicylcarbohydrazone (SACH) - SACH is a fluorescence dye that can be used for determination of Zn^{2+} at the micromolar concentrations (see Gonzalez *et al.*, 1984; de Torres *et al.*, 1990). It was synthesized by a one step organic Schiff base reaction involving the nucleophilic attack on carbohydrazide as described by Gonzalez *et al.* (1984). The reaction is shown in Figure 2-5A. SACH synthesis was as follows: carbohydrazide (0.9 g in 10 ml of water) was added to 2.6 ml of salicylaldehyde dissolved in 10 ml of 100% ethanol. The mixture was stirred in a 250 ml volumetric flask for about 30 sec and the volume was adjusted to 100 ml with 100% ethanol. This step iscritical as an insoluble precipitate forms if dilution time is delayed. The 100 ml mixture was then split into two 50 ml portions and incubated at 70°C for

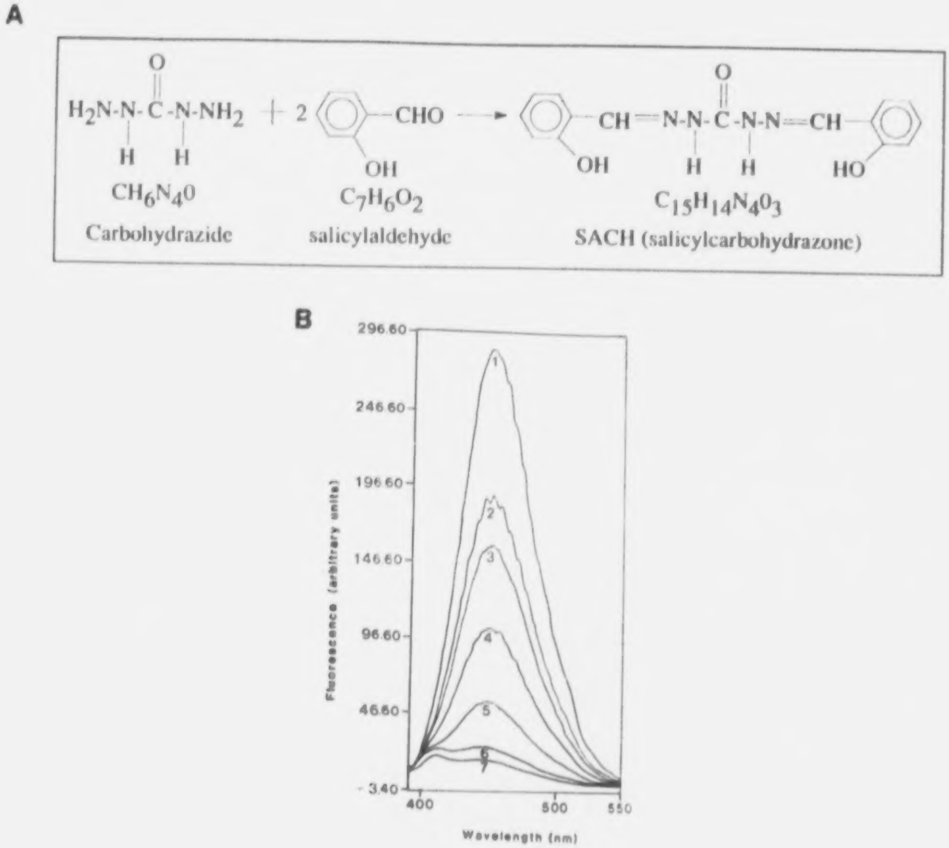


Figure 2-5: Synthesis and Zn^{2+}-dependent response of SACH

(A) SACH was synthesized by a one step organic Schiff base reaction involving the nucleophillic attack on carbohydrazide as described by Gonzalez *et al* .(1984).

(B) The dye has a fluorescence maximum at 448 nm and has a very good sensitivity to Zn^{2+} in a range from 0.6 μM to 800 μM. Curve 7, 0 μM; 6, 5 μM; 5, 20 μM; 4, 50 μM; 3, 75 μM; 2, 90 μM, 1, 150 μM Zn^{2+}.

30 min. with occasional stirring. The flasks were then transferred to room temperature and allowed to equilibrate for 30 sec. The yellowish supernatant was removed (20 ml at a time), immediately transferred to 4°C, and crystallized overnight at 4 °C. The crystals were dissolved in 15 ml of 100% ethanol at 70 °C followed by re-crystallization overnight at 4 °C. This step removes all impurities. The long yellow crystals containing SACH were dried down under a stream of nitrogen. The SACH crystals were subjected to melting point determination, NMR and mass spectroscopy. The melting point of SACH synthesized in our laboratory and used in this study was 228 °C and mass spectroscopy analysis indicated an average mass of 298.1063 mass units. These are in a good agreement with the reported values (molecular weight of 298.317 and a melting point of 235-236 °C). The NMR showed characteristic peak splitting as can be predicted based on the structure of SACH shown in Figure 2-5A. Average yields ranged from 40-60 mg of yellow crystalline dye (approximately 1.2 - 2%). The dye can be stored for months at -20 °C. Once dissolved, the dye is stable for only 24 hours.

Use of Zn^{2+} sensitive dye SACH - Zn^{2+} binding to the dye was carried out as described by de Torres *et al.* (1980) with some modifications. SACH was used at a final concentration of 50 µM. Endogenous divalent cations were removed from the samples used for Zn^{2+} analysis by dialyzing the samples for two days against a buffer containing 10 mM MOPS, 150 mM KCl, 1 mM EDTA (or 50 µM TPEN) followed by two more days of dialysis against a buffer containing 10 mM MOPS, 150 mM KCl. TPEN is the preferred choice of chelator because of its high affinity for

Zn^{2+} - $10^{15.58}$ M^{-1} vs $10^{4.4}$ M^{-1} for Ca^{2+}. A 1 mM stock solution of SACH was made in 100% ethanol. The assay was carried out in a total volume of 1 ml (900 µl of 50 µM SACH plus 100 µl of a solution containing 10 mM MOPS, pH 7.0 and 150 mM KCl) in the presence or absence of calreticulin (43 nM), S100a or calsequestrin (the latter two proteins were used as control Zn^{2+} binding proteins). Appropriate amounts of Zn^{2+} was added in 1 µl aliquots. Final concentrations were 24% ethanol, 0.16% Triton X-100, 16.4 mM acetic acid, and 3.6 mM sodium acetate. Zn^{2+} binding studies were also performed in the presence of 500 mM KCl to test the effect of non-specific binding resulting from electrostatic interactions. Measurements were carried out using a Shimadzu RF 5000U Spectrofluorophotometer. Samples were excited at 360 nm (band width 10 nm), and the emission spectrum was analyzed between 390 nm to 550 nm (band width 5.0 nm). The dye has a fluorescence maximum at 448 nm and has a very good sensitivity to Zn^{2+} in a range from 0.6 µM to 800 µM (Fig. 2-5B).

A calibration curve was obtained for the response of the dye to increasing amounts of Zn^{2+}. The calibration curve was prepared every time a new solution of the dye was made. The fluorescence values in the absence of protein (representing the free Zn^{2+} concentration) and in the presence of protein (representing the free and bound Zn^{2+} concentration) were converted to moles of Zn^{2+} (using the calibration curve) and then subtracted to obtain the amount of Zn^{2+} bound to the protein. The amount of bound Zn^{2+} can be divided by the amount of protein used to obtain the moles of Zn^{2+} bound per mole of protein (B value in Scatchard plots) and then divided by the amount of free Zn^{2+} to obtain the B/F value in Scatchard plots. K_d and B_{max} were obtained from a plot of B/F versus B.

$^{65}Zn^{2+}$ *overlay binding method* - $^{65}Zn^{2+}$ binding to proteins on nitrocellulose membranes was carried as described for $^{45}Ca^{2+}$ overlay by the method of Maruyama *et al.* (1984). Briefly, membranes were incubated and blocked for 4 hours in a solution containing 10 mM imidazole, pH 6.8, 5 mM $MgCl_2$, 60 mM KCl with changes every hour. The membrane was then incubated in the same buffer containing 100 μM $^{65}ZnCl_2$. $^{65}Zn^{2+}$ binding to proteins was visualized by autoradiography on Kodak X-OMAT AR films.

Zn^{2+}*-dependent precipitation* - Zn^{2+}-dependent precipitation of calreticulin was carried out in a buffer containing 10 mM MOPS, pH 7.1, 150 mM NaCl and different amounts of $^{65}ZnCl_2$. Precipitation was carried out for 16 hours followed by centrifugation for 45 min. at 100 000g. Pellet and supernatants were analyzed by SDS-PAGE. Calreticulin was identified by blue staining with Stains-All.

Zn^{2+} *Chelating Chromatography* - Zn^{2+}-IDA (iminodiacetate-substituted agarose) chromatography of purified proteins was carried out essentially as described by Porath and Olin (1983) and Picello *et al.* (1992). Zn^{2+}-IDA column (size - 8 cm x 1cm; flow rate - 0.5 ml/min) was first washed with a solution containing 50 mM NaH_2PO_4, pH 7.0, 100 mM NaCl and 5 mM EDTA followed by saturation of the column with 10 mM $ZnCl_2$. The excess of Zn^{2+} was removed by washing the column with a solution containing 50 mM NaH_2PO_4, pH 7.0 and 100 mM NaCl. Purified calreticulin or calreticulin domains were applied to the column, washed with 50 mM NaH_2PO_4, pH 7.0 and 100 mM NaCl and then eluted with 60 mls of a linear gradient of 0 to 50 mM imidazole in 50 mM NaH_2PO_4, pH 7.0 and 100 mM NaCl. Three ml fractions were collected and analyzed by SDS-PAGE. Flowthrough and eluted fraction were tested for reactivity with the goat anti-rabbit calreticulin and/or rabbit anti-bovine PDI antibodies.

Extraction of the GST-N-domain fusion protein

Frequently, overexpressed proteins accumulate in the cytoplasm in the form of insoluble inclusion bodies and account for a major fraction of the total expressed proteins in the E. coli sample (Lin and Cheng, 1991). The GST-N-domain fusion protein proved to be relatively insoluble and majority of the fusion protein was found in the 10 000g pellet after lysing of the E. coli cells. The insoluble GST-N-domain sample was extracted as described by Lin and Cheng (1991) (Fig. 3-6A). The pellet of the GST-N-domain sample (6 ml) was suspended in 50 ml of 20 mM Tris, 20% sucrose, 1 mM EDTA and incubated on ice for 10 min. The mixture was pelleted at 4,000g (5 875 rpm in a JA-17 rotor) for 10 min. and resuspended in 50 ml of ice cold water. This hypotonic treatment results in the lysis of the outer cell walls and was separated from the spheroplasts by centrifugation at 8,000g (7,725 rpm in a JA-17 rotor) for 10 min. The resultant pellet **(stage 1, Fig. 3-6, lane f)** was resuspended in 10 ml of a solution containing PBS, 5 mM EDTA, 0.5 ml protease inhibitors per 100 ml of solution, and 0.5 mM Benzamidine hydrochloride and PMSF (solution P) **(stage 2, Fig. 3-6, lane g)**. The mixture was sonicated 3 X 30 sec. (at a power output of 50 W) with a 30 sec. wait in between pulses. This treatment disrupted the cell membranes and released the protein content of the spheroplasts. Nonspecific protein interactions mediated via DNA or RNA molecules were disrupted by digestion at room temperature for 10 min. with RNase T1 (1300 U/ml of solution) and DNase 1 (40 µg/ml of solution). The solution was diluted by adding 40 ml of solution P and then pelleted at 13 000g (9750 rpm using a JA-17 rotor) for 30 min. The pellet **(stage 3, Fig. 3-6, lane h)** was resuspended in 40 ml of PBS, 25% sucrose, 5 mM EDTA, and 1% Triton-X100 (buffer W), incubated on ice for 10 min., and centrifuged at 25 000g (13 520 rpm in a JA-17 rotor) for 10 min. This was repeated two more times to wash the pellet. The pellet was resuspended in 10 ml of denaturation buffer containing 50 mM Tris-HCl, pH 8.0, 5 M guanidium chloride, and 5 mM EDTA **(stage 4, Fig. 3-6, lane i)** and sonicated for 5 sec. as before, followed by incubation on ice for 1 hour. This brief sonication step facilitated the solubilization of the aggregated proteins. The solubilized mixture was pelleted at 12 000g (9375 rpm using a JA-17 rotor) for 30 min., the pellet discarded **(stage 5, Fig. 3-6, lane j)** and the supernatant **(stage 6, Fig. 3-6, lane k)** resuspended in 100 ml of renaturation buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM DTT, 20% glycerol, 0.5 ml protease inhibitors per 100 ml of solution, and 0.5 mM Benzamidine hydrochloride and PMSF. The mixture was allowed to gently stir overnight at 4 ^{o}C to allow the protein to renature and assume a properly folded state. The sample was then clarified at 13 500g (10 000 rpm in a JA-17 rotor) for 30 min. and concentrated **(stage 7, Fig. 3-6, lane l)**. The solublized GST-N-domain was stored at - 20 ^{o}C until required for Ca^{2+} or Zn^{2+} binding analysis.

Electroelution of the N-domain of calreticulin

This domain of calreticulin is very hydrophobic and proved to be very difficult to purify. In order to obtain pure N-domain for functional analysis, extraction of the GST-N-domain fusion protein was carried out, followed by Factor Xa cutting of the extracted sample, followed by electroelution of the pure N-domain from the gel. Electroelution of the N-domain was carried as outlined by Anusbel *et al*, 1988 (pp. 10.5.1 -10.5.5). Proteins were first separated by SDS-PAGE (Laemmli, 1970), stained with Coomassie Blue, destained in methanol/acetic acid. The N-domain bands were cut out of the gel and allowed to soak overnight in water at 4 °C. Electroelution was carried out in a buffer containing 0.05 M NH_4HCO_3 containing 0.1% SDS (w/v) for 16 hours at 50 V (constant current). Dialysis of the electroeluted N-domain was carried out for 24 hours against a buffer containing 0.01 M NH_4HCO_3. The dialysate was lyophilized and stored at - 20 °C until needed.

Peptide Mapping using Stahylococcus Aureus (V8) digestion

Peptide mapping by limited proteolysis was performed by the method of Cleveland *et al.* (1977). Ten micrograms samples of purified proteins were resuspended in 20 µl of a buffer containing 0.125 M Tris-HCl pH 6.8, 0.5 % SDS, 10% glycerol, 0.0001 % bromophenol blue, ± 1 mM Ca^{2+}, ± 100 µM Zn^{2+}. The sample was heated for 2 minutes at 100 °C; V8 protease (1 : 20 enzyme to protein ratio) was added to a final concentration of 25 µg/ml and allowed to digest at 37 °C for 30 minutes. The reaction was stopped by the addition of 10 % 2-mecaptoethanol and by boiling for 2 minutes. Twenty-five microliters of the sample was separated by SDS-PAGE and then stained with Coomassie blue.

Diethyl pyrocarbonate (DEPC) modification of calreticulin

Modification of histidine residues in calreticulin was carried out as described by Miles (1977). Native or recombinant calreticulin (2 μM) were suspended in 50 mM sodium phosphate, pH 7.8 (50 mM sodium phosphate, pH 7.8 was filtered through a 0.22 μm filter). DEPC (BDH) was added in eight consecutive additions of a 35-fold molar excess of reagent with respect to calreticulin. DEPC was added from a 100 times concentrated solution in absolute ethanol. The ethanol was not allowed to exceed 2% in the final mixture. The reaction was carried out at 25 °C and was monitored by observing the change in absorbance at 243 nm. For each addition, readings were taken at the start of the DEPC addition and at 10 minutes after the addition. Modifications of histidine were estimated based on a differential extinction coefficient of 3 200 M^{-1} cm^{-1} at 243 for N-carbethoxyhistidine.

Reduction and alkylation of calreticulin

Native calreticulin was reduced using DTT and alkylated with iodoacetic acid. A 14 μM solution of calreticulin (approximately 2 mg) in 0.1 M Tris-HCl, pH 8.6 was added to 3.61 g urea. The volume was brought to 7.5 mls with water. The sample was flushed with N_2 and stirred at 25 °C for 30 min. An aliquot of 150 μl of a freshly prepared 0.1 M DTT was added to the solution, stirred and flushed again with N_2. The mixture was incubated at 37 °C for 90 min. An aliquot of 70 μl of a freshly prepared 0.5 M iodoacetic acid was added, the solution then flushed thoroughly with nitrogen, and then incubated in the dark at 37 °C for 40 minutes. The reaction was then terminated by lowering the pH to 3.0 with 12 M HCl and a drop of 2-mercaptoethanol. The solution was dialyzed extensively against double distilled, diionized water for two days at 4 °C. The sample was then lyophilized and then used for Zn^{2+} analysis using SACH.

Carbohydrate analysis

Endoglycosidase digestion of calreticulin was carried out as described by Michalak *et al.* (1990) with some modifications. Twenty micrograms of calreticulin was freeze dried and resuspended in 20 mM sodium phosphate, pH 6.1, 50 mM EDTA, 1% β-mercaptoehtanol and 0.1% SDS. The mixture was incubated at 37 °C for 30 min. in the presence of either endoglycosidase H (0.1 mU/μg protein), endoglycosidase D (5 mU/μg protein), endoglycosidase F (5 mU/μg protein) or glycopeptidase F (18 mU/μg protein). Incubation was allowed to continue for 16 h at 37 °C. Sample buffer (20 % glycerol, 4% SDS, 0.130 M Tris-HCl, pH 6.8, 0.001% bromophenol blue) was added and the samples were analyzed by SDS-PAGE. Analysis of total carbohydrate content of calreticulin was estimated by the phenol/H_2SO_4 method of Dubois *et al.*, (1956). In brief, 0.5 ml of each protein sample (1 mg/ml) was mixed with 0.1 ml of 5 % phenol (w/v). After thorough mixing by vortexing, the color was developed by the slow addition of 0.5 ml of concentrated H_2SO_4. The samples were incubated at 50 °C for 20 minutes and, after cooling, the A_{490} was measured. Glucose (100 μg/ml) was used as a standard.

Protein Kinase Assays

Two hundred micrograms of calreticulin or control Histone IIIs (Sigma Chemicals, Inc.) were used with 10 μg of protein kinase C (Sigma Chemicals, Inc.) (1 : 20 dilution of enzyme to substrate). The assay was carried out in 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 180 μM $CaCl_2$, 0.25 mM phosphatidylserine and 64 μM 1, 2 - diolein (mixed micellar phospholipid requirement for protein kinase C purchased from Serdary Research), and 100 000 dpm/μl of γ-^{32}P-ATP. The ATP was added after the mixture of protein and assay buffer was allowed to incubate for 2 min. at 30 ^{o}C, followed by addition of γ-^{32}P-ATP. At different times, the reactions were stopped by adding the 100 μl aliquots to 1 ml of 25 % trichloroacetic acid (TCA), and the mixture was spun down for 3 min. at (12 000g) 14 000 rpm in an eppendorf centrifuge. The TCA precipitable proteins were analyzed by SDS-PAGE.

Disulfide bond analysis

Disulfide bond analysis was carried using the Ellman's reagent (Sigma) - 5,5′-Dithio-*bis*-(2-nitrobenzoic acid) (DTNB). Calreticulin (100 - 200 μg) was resuspended in 0.1 M Na_2HPO_4, pH 8.0. Ellman's reagent (4 mg/ml) was dissolved in 0.1M Na_2HPO_4, pH 8.0. The reaction was initiated by addition of 100 μl of protein sample to 100 μl of Ellman's reagent and 5 ml of 0.1M Na_2HPO_4, pH 8.0. The mixture was incubated for 15 min. and changes in the absorbance were monitored at 412 nm. L-cysteine (Sigma) was used as a standard. The molar extinction coefficient of DTNB at 412 nm is 1.36×10^4 $cm^{-1}M^{-1}$.

Sedimentation equilibrium analysis

Analytical Ultracentrifuge Experiment - A Beckman Model E Analytical Ultracentrifuge equipped with electronic speed control, RTIC temperature control system, titanium rotor, and photoelectric scanner absorbance optical system was used for both the sedimentation equilibrium and sedimentation velocity experiments.

Determinations of molecular weights and sedimentation coefficients were performed using the methodology described by Chervenka (1969). Samples were dialyzed for 48 hours at 4 °C prior to running in the ultracentrifuge. All runs were performed at 20 °C using a speed of 15 000 rpm. An assumed value of 0.73 was used for the partial specific volume.

For molecular weight determinations, 100 µl of sample solution were loaded into the right side of a double-sector, CFE sample cell equipped with sapphire windows. One hundred and five µl of dialysate were loaded into the left sector. Samples were run for a minimum of 48 hours prior to taking equilibrium traces. Molecular weight calculations were carried out using an APL computer program. The lny vs r^2 data were fitted to a 2nd order polynomial equation and the apparent weight average molecular weight was calculated from the slope of the resulting plot.

Sedimentation velocity experiment - The similar type of sample cell to that used for the sedimentation equilibrium run was used for the sedimentation velocity run, with 350 µl of sample solution loaded into the right side and 355 µl of dialyzate in the left side. The rotor was accelerated to 60 000 rpm, and scanner traces were taken at regular time intervals as the protein sedimented to the cell bottom. The midpoint of the trailing boundary region in each trace was used to monitor the sedimentation of the protein. The S_{obs} was calculated from the center of the rotor. The S_{obs} was then corrected to standard conditions (water at 20 °C) to obtain $S_{20,w}$.

Both sedimentation equilibrium and velocity measurements were carried out in 150 mM KCl, 50 mM MOPS, 1 mM EDTA, 2 mM DTT (pH 7.1) using a protein concentration of 0.3 - 0.4 mg/ml.

Circular Dichroism Analysis

Circular dichroism (CD) measurements were made on a J-720 spectropolarimeter (Jasco, Inc., Easton, MD) interfaced with an Epson Equity 386/25 computer. The cell was maintained at 25 °C with an RMS circulating water bath (Lauda, Westbury, N.Y.). Near-uv (320-250 nm) scans were performed in a microcell of path length 1 cm, volume 90 µl. In the wavelength range 255 to 190 nm, cells of path length 0.01 or 0.02 cm were used. The computer-averaged trace of 10 scans was used in all calculations. Signal due to solvent was subtracted. The instrument was routinely calibrated with *d*-(+)-10-camphorsulfonic acid at 290 nm and with pantoyl lactone at 219 nm by following procedures outlined by the manufacturer. The data were normally plotted as mean residue weight ellipticity (expressed in degrees square centimeters per decimole) versus wavelength in nanometers. The mean residue weight was taken to be 116. Secondary structure analysis was performed using the algorithm of Provencher and Glöckner (1981).

Peptide Synthesis

Peptides 083 and 0811 were synthesized using the general procedure for solid-phase synthesis described by Hodges *et al.* (1988). Peptide synthesis was carried out on an Applied Biosystems peptide synthesizer Model 430A (Foster City, CA) using the copolymer poly(styrene-1% divinylbenzene)benzhydrylamine hydrochloride resin (0.92 mmol of NH_2/g). After the NH_2-terminus was acetylated with 25% acetic anhydride/dichloromethane (v/v), the peptides were cleaved from the resin by reaction with hydrogen fluoride (20 ml/g of resin) containing 10% anisole and 2% 1,2-ethanedithiol for 1 hour at - 4 ^oC. The crude peptides were extracted with 30% acetic acid and lyophilized. Peptide purification was carried out by HPLC using a semipreparative reversed-phase C18 column (Synchropak RP-P, 250 x 10 mm inner diameter, 6.5 µm particle size, 300 Å pore size, SynChrom, Lafayette, IN). The peptides were eluted with a linear AB gradient (ranging from 0.2% to 2% B/min, depending of the retention time of the peptide) at a flow rate of 2 ml/min, where solvent A was 0.05% trifluoroacetic acid in water and solvent B was 0.05% trifluoroacetic acid in acetonitrile. The purity and identity of the peptide in the fractions were verified using a Hewlett-Packard HP 1090 HPLC equipped with an analytical reversed-phase C8 column (Zorbax 300SB-C8, 25 cm x 4.6 mm, 5 µm particle size, 300 Å pore size) using as linear AB gradient of 2% B/min. Fractions were pooled and lyophilzed. The purified peptides were homogenous as determined by analytical reversed-phase HPLC, amino acid analysis, and mass spectrometry.

Protein Sequencing

NH_2-terminal amino acid sequence analysis was carried out on proteins electroblotted to Immobilon (polyvinylidene difluoride) membrane (Matsudaira, 1987). Automated sequence analyses (Hewick *et al.*, 1981) were performed with an Applied Biosystems Model 470A gas-liquid phase protein sequencer connected on-line to an Applied Biosystems Model 120A HPLC, using current protocols of Applied Biosystems. All sequencer chemicals were from Applied Biosystems, Foster City, CA. Protein sequencing was carried out at the Alberta Peptide Institute, University of Alberta, AB and in the Department of Biochemistry, University of Victoria, BC.

Immunofluorescence Microscopy

For intracellular localization of calreticulin and calsequestrin polyclonal antibodies were used at 1:50 and 1:30 dilution in PBS, respectively. FITC-conjugated secondary antibodies (used at 1:30 dilution in PBS) were purchased from ICN ImmunoBiologicals (Montreal, Que.). For fluorescence microscopy, cells were fixed in 3.8% formaldehyde in PBS for 10 min, extracted with 0.1% Triton X-100 in a buffer containing 100 mM PIPES, 1 mM EGTA, 4% (w/v) poly(ethylene glycol) 8000 (pH, 6.9) for 3 min, washed in PBS for 10 min, and then processed for labeling with primary antibodies followed by appropriate FITC-conjugated secondary antibodies. All incubations were carried out for 30 min at room temperature, followed by a 10 min wash with PBS. After final wash, the slides were mounted in Vinol 205S (St. Lawrence Chemical, Toronto, Ont.), which contained 0.25% 1,4-diazobicyclo-(2,2,2)-octane (Polysciences) and 0.002% *p*-phenylenediamine (Fisher Scientific) to prevent photobleaching. Confocal fluorescence microscopy was performed by Dr. Michal Opas at the Ontario Laser and Lightwave Research Center using a Bio-Rad MRC-600 microscope.

SDS-Polyacrylamide Gel Electrophoresis and Protein Standards-

SDS-PAGE was on 10%, 12.5%, or 15% polyacrylamide gels as described by Laemmli (1970). After gel electrophoresis, gels were stained with either Coomassie Blue or stained with the carbocyanine dye "Stains-All" as described by Campbell *et al.* (1983); or transferred electrophoretically onto nitrocellulose membrane (Towbin *et al.*, 1979).

Standards were Bio-Rad low range molecular weight proteins; phosphorylase b (97 400), bovine serum albumin (66 200), ovalbumin (42 700), bovine carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500) and lysozyme (14 400), or Bio-Rad low range prestained markers; phosphorylase b (106 000), bovine serum albumin (80 000), ovalbumin (49 000), carbonic anhydrase (32 000), soybean trypsin inhibitor (27 000) and lysozyme (17 000). Very low range standards were from Mandel Scientific; carbonic anhydrase (29 000), trypsin inhibitor (20 400), lysozyme (14 000), aprotinin (6 100), and insulin chain B (3 500).

Determination of protein concentration

Protein was determined by the method of Lowry *et al.* (1951) or Bradford (1976). Bovine serum albumin was used a standard in these assay.

CHAPTER THREE

Purification, expression, and biochemical characterization of calreticulin and calreticulin domains

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I. Purification and Expression of calreticulin and calreticulin domains

INTRODUCTION

In order to carry out biochemical studies on calreticulin I have developed a standard method for the rapid and easy purification of the native and recombinant protein. Calreticulin was first purified by MacLennan's group and referred to as "the high affinity Ca^{2+}-binding protein" (Ostwald and MacLennan, 1974; Michalak *et al.*, 1990; and MacLennan, 1984). MacLennan's group used isolated SR vesicles as the starting material for the purification of the protein and calreticulin was extracted from the lumen of membrane vesicles with a low concentration of deoxycholate, in the presence of 1 M KCl, followed by gel filtration, DEAE cellulose and hydroxylapatite chromatography (MacLennan, 1974). Waisman's group (Waisman *et al.*, 1985) used liver homogenates for the purification of calreticulin ("calregulin"). Their procedure, however, required freeze-thawing of the liver in order to release membrane-bound calreticulin followed by ion exchange chromatography.

The first part of my research project was to develop an efficient method for the isolation of native calreticulin and prokaryotic expression and isolation of recombinant calreticulin and its domains. We have used the canine pancreas as a source of native calreticulin based on the observation that this tissue is one of the richest sources of calreticulin (Khanna *et al., 1986*). Calreticulin is a peripheral membrane protein and could be extracted from the microsomal membrane with carbonate buffer at pH 11.4 (Michalak *et al.*, 1991). It was identified in the membrane fractions by immunoblotting with a specific antibody, by a $^{45}Ca^{2+}$ overlay technique and by NH_2-terminal amino acid analysis of the purified protein. This chapter will detail efficient procedures for the isolation of native calreticulin from canine pancreas, and the isolation of recombinant calreticulin and calreticulin domains expressed in E. coli as a Glutathione S-transferase fusion proteins. The recombinant proteins were purified by Glutathione-Sepharose affinity chromatography and Mono Q anion exchange FPLC chromatography. These protocols require only commercially available materials and yield the protein in sufficient quantities to carry out comprehensive biochemical, functional, and structural characterization studies.

RESULTS

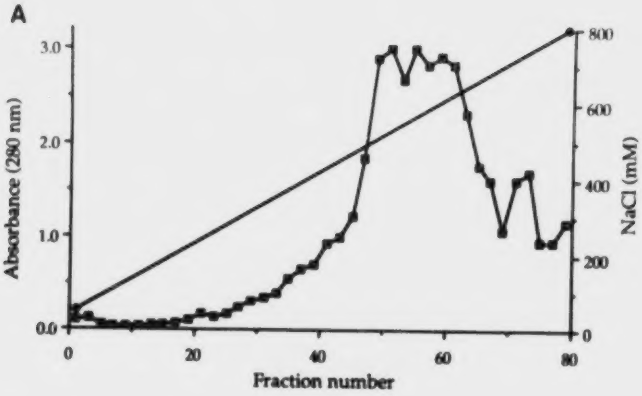
Purification of Native Calreticulin

Native calreticulin was isolated by a selective ammonium sulfate precipitation procedure. This method involved direct extraction of the protein from whole tissue homogenates and eliminated the need to prepare membrane fractions. This procedure was based on the method developed for the isolation of muscle calsequestrin (Slupsky *et al.*, 1987) and takes advantage of the high solubility of calreticulin in an ammonium sulfate solution (Michalak *et al.*, 1980). Crude tissue homogenates were extracted with 55% saturated ammonium sulfate, which precipitated most cellular proteins, followed by precipitation of calreticulin with 85% saturated ammonium sulfate at pH 4.7 (approximately at the pI point of the protein). Use of protease inhibitors throughout the purification was mandatory since proteolysis of calreticulin was a problem. Pure calreticulin was obtained from the 85% ammonium sulfate precipitate by DEAE-Sephadex chromatography followed by hydroxylapatite column chromatography. This was similar to chromatography methods used by MacLennan's group to purify calreticulin from isolated SR vesicles (Ostwald and MacLennan, 1974; MacLennan, 1974).

Figure 3-1 shows the absorbance profile (A) and SDS-PAGE (B) of fractions obtained from a DEAE-Sephadex column. Calreticulin eluted from this column in a broad peak at approximately 300 - 400 mM NaCl (Fig. 3-1A and B, arrow). A major contaminant co-eluting with calreticulin was a 100-kDa protein (Fig. 3-1B, closed triangle), identified by NH_2-terminal amino acid sequence analysis to be endoplasmin or Grp94 (Maki and Srivastava, 1990; Garrigos *et al.*, 1991) (D-D-E-V-D-V). DEAE-Sephadex fractions containing calreticulin were pooled (fractions # 31 - 47), dialyzed and entire sample was then applied onto a hydroxylapatite column (Fig. 3-2A and B). Calreticulin eluted at 213 mM potassium phosphate and was resolved from the contaminating proteins (Fig. 3-2A and B, arrow). Fractions # 15 - 17 were pooled from hydroxylapatite column and they contained pure native calreticulin. Analysis of the purified native calreticulin by reverse phase HPLC chromatography revealed only one protein peak, which eluted at the same retention time (60 min) as the recombinant protein (Fig. 3-2C, compare to Fig. 3-4B). The identity of native calreticulin was confirmed by the NH_2-terminal amino acid sequence analysis of the protein (E-P-A-I-Y-K), by its cross reactivity with anti-calreticulin antibodies and by its Ca^{2+} binding (see below). The

Figure 3-1: DEAE-Sephadex A-50 purification of native calreticulin.

DEAE-Sephadex chromatography was carried out as described under "Chapter Two: Experimental Procedures". (A) Absorbance at 280 nm; (B) SDS-PAGE (10% gel) of fractions containing calreticulin from the DEAE-Sephadex column. Lane A, 85% ammonium sulfate precipitate; lane B, flow-through (unbound protein); lanes 1 - 47, selected protein fractions from DEAE-Sephadex column (see A). Fifteen ml samples of each of the 1 ml fractions were separated by SDS-PAGE. The arrow indicates position of calreticulin; closed triangle indicates position of Grp94 (endoplasmin).


A
Absorbance (280 nm)
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NaCl (mM)
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800
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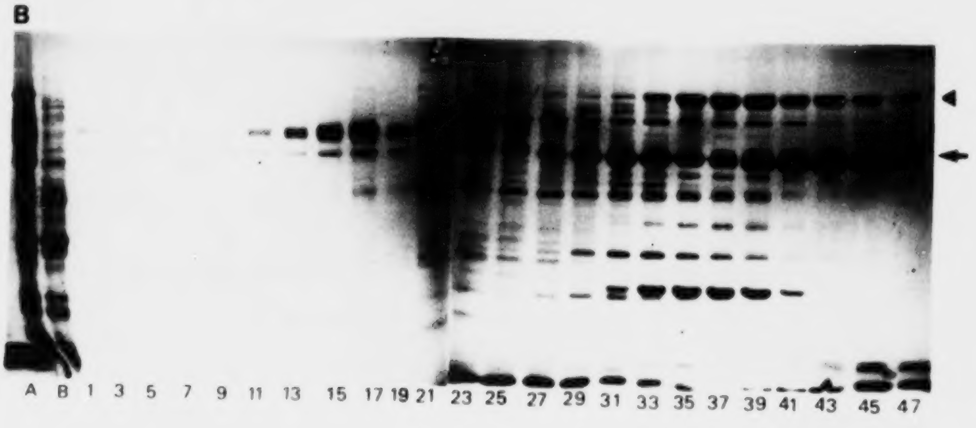
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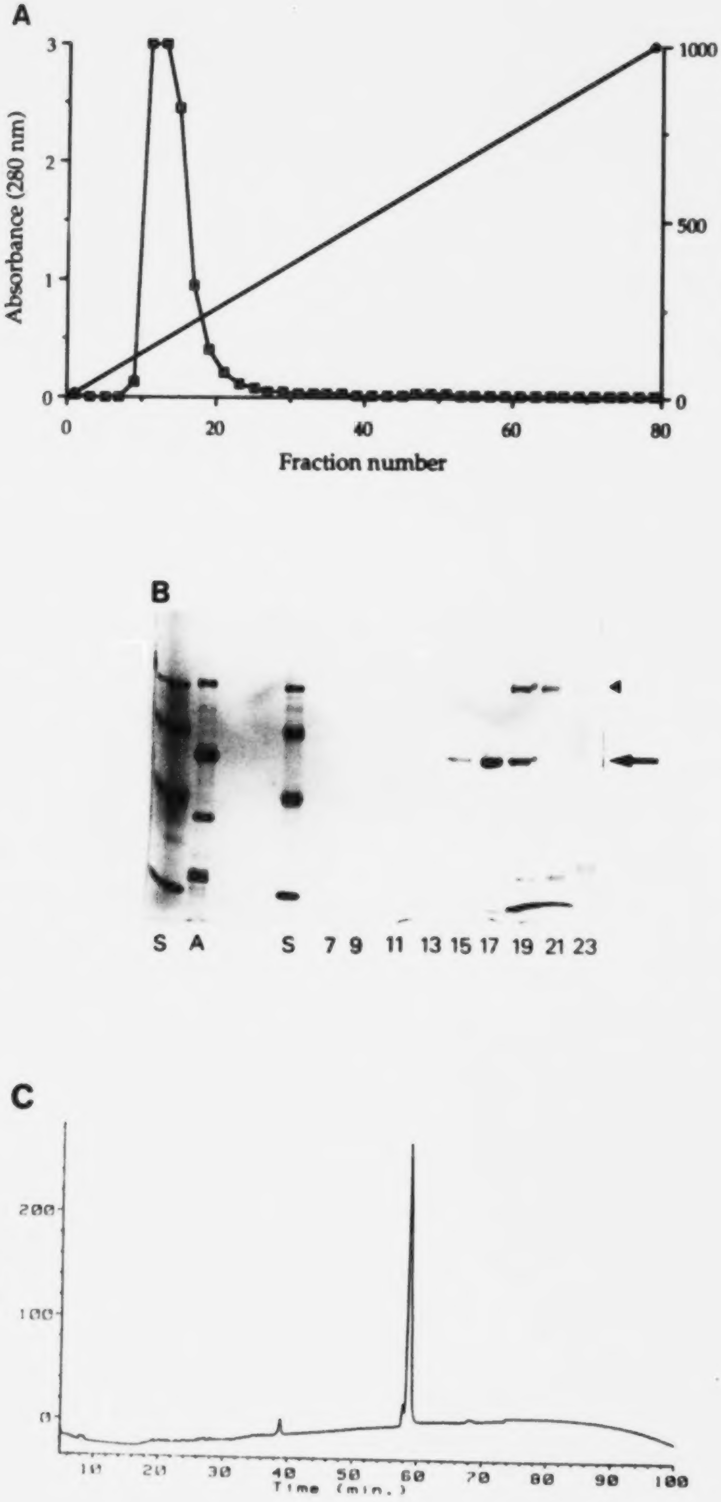
47

Figure 3-2: Hydroxylapatite chromatography of calreticulin.

(A, B) DEAE-Sephadex fractions containing calreticulin were pooled, dialyzed and applied onto a freshly prepared hydroxylapatite column as described under "Chapter Two: Experimental Procedures". (A) Absorbance at 280 nm. (B) SDS-PAGE (10% gel) of fractions containing calreticulin. Lane A, DEAE-Sephadex purified calreticulin; Lanes 7 - 23, protein fractions from the hydroxylapatite column (see A). Fifteen ml samples of each of the 1 ml fractions were separated by SDS-PAGE. Lane S, molecular weight marker proteins phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (42,700), bovine carbonic anhydrase (31,000) and soybean trypsin inhibitor (21,500). The arrow indicates position of calreticulin; closed triangle indicates position of Grp94 (endoplasmin).

(C) HPLC reverse phase chromatography of native calreticulin is shown. Purified native calreticulin (fraction 17 of B) was applied to a C18 narrow bore reverse phase column. Chromatography was carried out at 1% B/min (A = 0.05% TFA/H_2O; B = 0.05% TFA/acetonitrile).


A
Absorbance (280 nm)
3
2
1
0
Potassium phosphate (mM)
1000
500
0
0
20
40
60
80
Fraction number
B
S
A
S
7
9
11
13
15
17
19
21
23
C
mAU
200
100
0
10
20
30
40
50
60
70
80
90
100
Time (min.)

major contaminant 100-kDa protein co-eluting with calreticulin (Fig. 3-2B, closed triangle), was assumed to be endoplasmin and was clearly separated from the calreticulin peak.

Figure 3-3A, B, and C characterized the enrichment of native calreticulin throughout the various stages of its purification. As shown in Fig. 3-3A, lane 4 native calreticulin samples frequently, but not always, split into two closely migrating bands. When EGTA was included in all SDS-PAGE buffers calreticulin migrated as one band, indicating that Ca^{2+} binding to the protein may be changing its electrophoretic mobility in Laemmli SDS-PAGE. Similar Ca^{2+}-induced changes in the mobility in SDS-PAGE were also observed for muscle calsequestrin (Garrigos *et al.*, 1991). Calreticulin was identified throughout the purification procedure by immunostaining with goat anti-calreticulin antibodies (Fig. 3-3B) and by Ca^{2+} binding by a $^{45}Ca^{2+}$ overlay (Fig. 3-3C). Anti-calreticulin antibodies specifically recognized a 60-kDa band (Fig. 3-3B, arrow) at all stages of purification, and native canine pancreatic calreticulin bound Ca^{2+} in a $^{45}Ca^{2+}$ overlay technique (Fig. 3-3C). Table 3-1 numerically illustrated the enrichment of native calreticulin throughout the various stages of its purification. Routine purification of calreticulin from three canine pancreas yielded between 5 - 8 mg of protein that remained stable (that is, not degraded) at - 20 °C for 6 - 8 months. If stored longer than 6 - 8 months, the protein degraded to a stable 43 kDa protein band that was still immunoreactive with the goat anti-calreticulin antibody but no longer reactive with a KDEL-specific antibody. It thus appeared that a significant portion of the carboxyl-terminus of the protein was missing in this degraded form of the protein. The key stage in the isolation procedure

Figure 3-3: SDS-PAGE summary of the purification of native and recombinant calreticulin.

Native and recombinant calreticulin were purified as described under "Chapter Two: Experimental Procedures". A,D - Coomassie blue staining; in B,E and C,F proteins were separated by SDS-PAGE (12.5 % gel), transferred to nitrocellulose filters and incubated with goat anti-calreticulin (B,E) or incubated with $^{45}Ca^{2+}$ (C,F) as described under "Experimental Procedures".

(A, B, C) - Samples are: Lane 1, 55% ammonium sulfate precipitate; lane 2, 85% ammonium sulfate precipitate; lane 3, DEAE-Sephadex purified calreticulin (pooled fractions # 31- 47; see Fig. 3-1); lane 4, hydroxylapatite purified native calreticulin (pooled fractions # 15- 17; see Fig. 3-2); lane 5, Mono Q purified recombinant calreticulin (see fig. 3-4, fraction 24). S, molecular weight markers which are indicated. The arrows indicate position of calreticulin.

(D, E, F) Samples are: Lane 1, purified canine pancreatic calreticulin; lane 2, Glutathione-Sepharose purified recombinant GST; lane 3, E. coli lysate containing GST-calreticulin fusion protein; lane 4, Glutathione-Sepharose purified GST-calreticulin fusion protein; lane 5, factor Xa cleaved GST-calreticulin fusion protein; lane 6, Mono Q FPLC purified recombinant calreticulin. S, Bio-Rad low molecular weight standards. In F prestained molecular weight markers were used. The positions of molecular weight marker proteins are indicated. The appearance of the Mr standards in F is not due to Ca^{2+} binding to these proteins.

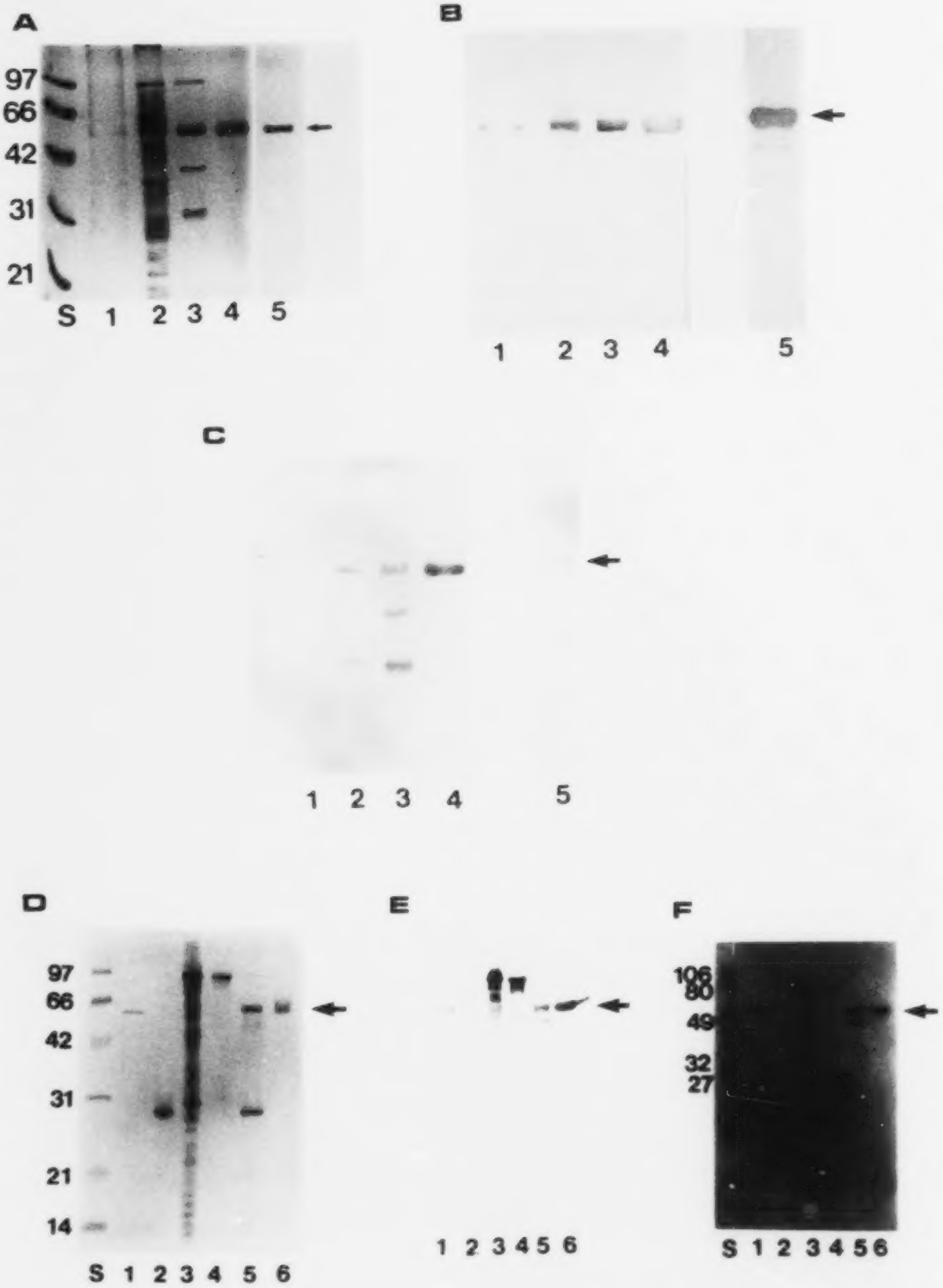

A
97
66
42
31
21
S 1 2 3 4 5
B
1 2 3 4 5
C
1 2 3 4 5
D
97
66
42
31
21
14
S 1 2 3 4 5 6
E
1 2 3 4 5 6
F
106
80
49
32
27
S 1 2 3 4 5 6

Table 3-1:

Purification of Native Calreticulin from Canine Pancreas

Fraction	Vol (ml)	Protein (mg/ml)	Total Protein (mg)	Yield % *1
55% ammonium sulfate supernatant	450	1.04	468.0	100
85% ammonium sulfate pellet	70	5.2	364.0	77.8
DEAE-Sephadex fraction	10	2.16	21.6	4.6
Hydroxylapatite fraction	4	1.27	5.08	1.1

Note. Details of the purification procedure are in "Chapter Two: Experimental Procedures". DEAE-Sephadex and Hydroxylapatite fractions correspond to the pooled fractions #31 - 47 and #15 - 17 (see Fig. 3-1 and 3-2, respectively).

*1 This represents percent of the 55% ammonium sulphate precipitable fraction.

was the use of fresh hydroxylapaptite beads that greatly improved the yield of the protein at the last stage of the isolation protocol.

Expression and Purification of Recombinant Calreticulin

Protein characterization studies sometimes require significant amounts of protein that are not usually available when isolated from tissue sources. In order to resolve this dilemma, recombinant technology was employed to obtain workable quantities of the protein for biochemical analysis. Recombinant calreticulin was expressed in the GST prokaryotic expression system to yield a chimeric protein of GST and calreticulin. SDS-PAGE of recombinant calreticulin expressed in *E. coli* as a fusion protein with GST is shown in Fig. 3-3D. Upon lysis of the cells by French pressure the recombinant GST-calreticulin fusion protein was a major component of the *E. coli* soluble extract (Fig. 3-3D, *lane 3, see arrow*). The solubility of the fusion protein suggested that it was correctly folded within *E. coli* and might, therefore, be able to bind Ca^{2+}. Approximately 30 µg of the GST-calreticulin fusion protein was obtained per ml of *E. coli* culture, of which over 90% was purified by one-step Glutathione-Sepharose column chromatography (Fig. 3-3D, *lane 4*).

The GST-calreticulin fusion protein purified by affinity column chromatography migrated in SDS-PAGE with a mobility corresponding to a polypeptide of 80-kDa (Fig. 3-3D, *lane 4*). This mobility does not correspond to the predicted size of the fusion protein (72-kDa; 46-kDa calreticulin + 26-kDa recombinant GST). The anomalous mobility of the fusion protein is reminiscent of the anomalous mobility of native calreticulin in SDS-PAGE (60-kDa in Laemmli SDS-PAGE vs a molecular weight of 46,600 predicted for the protein from its amino acid sequence) (Krause *et al.*, 1990; Milner *et al.*, 1991).

The pGEX-3X plasmid encodes GST (26-kDa) (Fig. 3-3D, lane 2) followed immediately by a Factor Xa recognition site. Therefore, the fusion protein can be cleaved with Factor Xa and the recombinant calreticulin protein purified. SDS-PAGE of the GST-calreticulin fusion protein after cleavage by Factor Xa, is shown in Fig. 3-3D, lane 5. Under the conditions used complete cleavage of the fusion protein was obtained. Full length calreticulin-GST fusion protein and Factor Xa cleaved recombinant calreticulin, as expected, cross-reacted with goat anti-rabbit calreticulin antibodies (Fig. 3-3E), whereas recombinant GST did not (Fig. 3-3E, lane 2). The purified GST-calreticulin fusion protein (Fig. 3-3F, lane 4) and factor Xa cleaved recombinant calreticulin (Fig. 3-3F, lane 5) all bound Ca^{2+} by $^{45}Ca^{2+}$ overlay technique. Recombinant GST did not bind any Ca^{2+} in either $^{45}Ca^{2+}$ overlay (Fig. 3-3F, lane 2) or equilibrium dialysis.

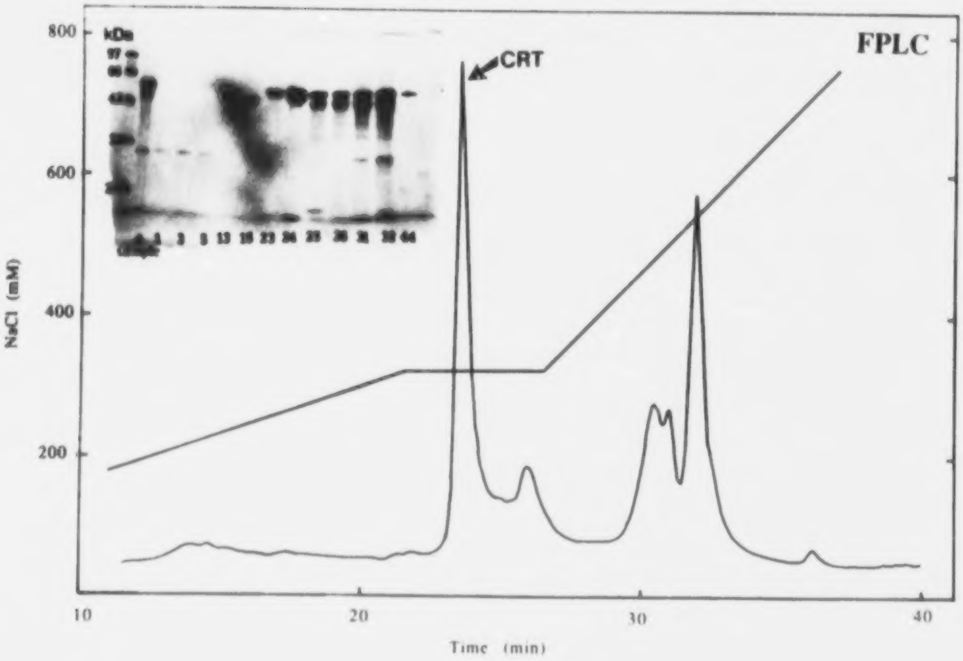
Recombinant calreticulin was separated from GST by chromatography on a Glutathione-Sepharose affinity column, followed by Mono Q FPLC. Pure recombinant calreticulin was obtained by Mono Q FPLC chromatography (Fig. 3-4A). The protein eluted in a relatively sharp peak at approximately 300 mM NaCl (Fig. 3-4A) followed by peaks containing both calreticulin and other contaminating proteins. Analysis of the Mono Q FPLC purified recombinant calreticulin by reverse phase HPLC chromatography revealed only one protein peak indicating that a high level of purity was achieved (Fig. 3-4B). The identity of recombinant calreticulin was confirmed by NH_2-terminal amino acid sequence

Figure 3-4: Mono Q FPLC chromatography of Glutathione-Sepharose purified recombinant calreticulin.

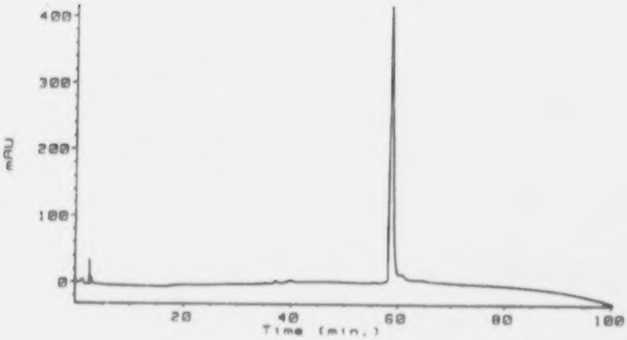
(A) Recombinant calreticulin was purified by Glutathione-Sepharose affinity chromatography, cleaved with factor Xa, passed over a second Glutathione-Sepharose column and purified by Mono Q FPLC chromatography as described under "Chapter Two: Experimental Procedures". The protein was applied to an FPLC Mono Q 5/5 anion exchange column followed by elution with a step NaCl gradient (50 - 300 mM NaCl; and 300 - 750 mM NaCl) . One milliliter fractions were collected and absorption at 280 nm and NaCl concentrations were monitored. SDS-PAGE (12.5 % gel) of the starting material ("sample") and of peak fractions is shown as an inset. The positions of the calreticulin peak and protein band are indicated by the arrow. CRT, calreticulin.

(B) HPLC reverse phase chromatography of recombinant calreticulin is shown. Recombinant calreticulin (fraction 24 of A) purified by Mono Q FPLC chromatography, was applied to a C18 narrow bore reverse phase column. Chromatography was carried out at 1% B/min (A = 0.05% TFA/H_20; B = 0.05% TFA/acetonitrile).

A


FPLC
CRT
kDa
97
1
3
5
13
15
23
24
25
26
31
32
44
NaCl (mM)
800
600
400
200
10
20
30
40
Time (min)

B


mAU
400
300
200
100
0
20
40
60
80
100
Time (min.)

analysis of the purified protein. The NH_2-terminal amino acid sequence of the recombinant calreticulin was NH_2-G-I-P-G-N-S-E-P-V-V-Y-F-K-E-Q-F-, where the underlined amino acid sequence corresponded to the amino acid sequence of mature calreticulin (Fliegel *et al.*, 1989b). The first six NH_2-terminal amino acid residues of the recombinant calreticulin (NH_2-G-I-P-G-N-S-) corresponded to the translation of the BamH1/Sma1/EcoR1 nucleotide sequence which was part of the multiple cloning site in pGEX-3X (Smith and Johnson, 1988).

Both native calreticulin and the recombinant protein eluted at the same salt concentration from the Mono Q FPLC column (0.3 M NaCl) (Fig. 3-1 and Fig. 3-4, respectively), and both proteins had the same mobility in SDS-PAGE, corresponding to an apparent molecular weight of 60 000 (Fig. 3-4A, lanes 4 and 5). The amino acid composition of recombinant and native calreticulins was virtually identical, confirming the identity of these calreticulin samples. Furthermore, both proteins cross-reacted with goat anti-calreticulin antibodies (Fig. 3-4B, lanes 4 and 5) and bound Ca^{2+} in a $^{45}Ca^{2+}$ overlay technique (Fig. 3-4C, lanes 4 and 5).

The Expression of Distinct Domains of Calreticulin as Fusion Proteins

In order to characterize Ca^{2+} binding to calreticulin, and to identify specific domains in the protein that might be responsible for Ca^{2+} binding we utilized a fusion protein approach. Structurally, calreticulin can be divided into three distinct domains (Fliegel *et al.*, 1989b; Smith and Koch, 1989) (Fig. 3-5A): the N-domain containing the first 182 amino acids, the carboxyl-terminal quarter of the protein (residues 270 - 401) (C-domain), and the sequence connecting these two regions which is

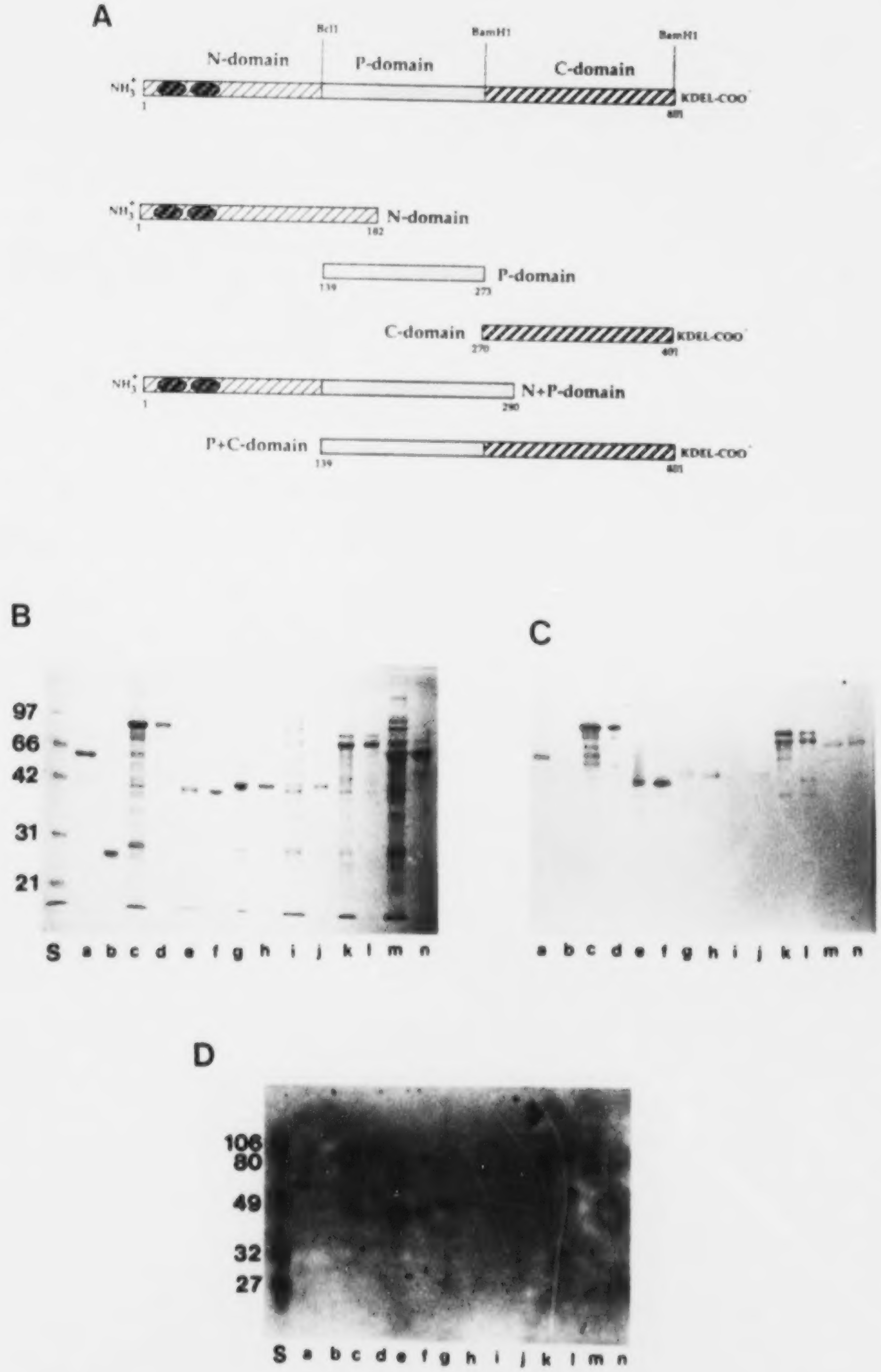
Figure 3-5:

Putative domain organization of calreticulin

(A) The amino acid sequence of the mature rabbit skeletal muscle calreticulin in Fig 1-2A was divided into specific domains, which were subcloned into unique restriction sites in the pGEX-3X plasmid as described under "Chapter Two: Experimental Procedures." The residue numbers correspond to the residue numbers in Fig. 1-2A. P-, C- and P+C-domains were subcloned as restriction fragments. The N- and N+P-domains were subcloned using PCR technology. Two ovals in the N-domain depict the approximate location of the two predicted helices in the NH_2-terminal end of the protein (Fig. 1-2A).

Expression, purification and characterization of calreticulin fusion proteins.

(B, C, D) E. coli lysates and purified proteins were separated by SDS-PAGE (12.5 % gel), transferred electrophoretically to nitrocellulose membranes and incubated with anti-calreticulin antibody, or with $^{45}Ca^{2+}$ as described under "Chapter Two: Experimental Procedures." B, Coomassie Blue staining; C, immunostaining with goat anti-calreticulin antibody; D, autoradiography of B after electrophoretic transfer and incubation with $^{45}Ca^{2+}$. Lane a, purified pancreatic calreticulin; lane b, Glutathione-Sepharose purified recombinant GST; lanes c, e, g, i, k, and m, E. coli lysates containing fusion proteins; lane d, f, h, j, l and n, Glutathione-Sepharose purified fusion proteins. Lanes c and d, full length recombinant calreticulin; lanes e and f, C-domain; lanes g and h, P-domain; lanes i and j, N-domain; lanes k and l, P+C-domain; lanes m and n, P+N-domain. S, Bio-Rad low molecular weight standards in B and prestained Bio-Rad low molecular weight markers in D. The positions of molecular weight marker proteins are indicated. The appearance of the Mr standards in D is due to the light emission from the dye bound to the standards proteins.


A
BclI
BamH1
BamH1
N-domain
P-domain
C-domain
NH3+
KDEL-COO-
1
401
NH3+
N-domain
1
182
P-domain
139
273
C-domain
270
401
KDEL-COO-
NH3+
N+P-domain
1
290
P+C-domain
139
401
KDEL-COO-
B
97
66
42
31
21
S a b c d e f g h i j k l m n
C
a b c d e f g h i j k l m n
D
106
80
49
32
27
S a b c d e f g h i j k l m n

enriched in proline residues (residue 139 - 273) (P-domain). The following domains were expressed in E. coli as GST fusion proteins: N-domain, P-domain, C-domain, P+C-domain, and N+P-domain (Fig. 3-5A, details on the construction of the recombinant molecules are under "Chapter Two: Experimental Procedures").

GST fusion proteins were expressed at high levels in BNN103 E. coli after induction with IPTG (Fig. 3-5B, lanes c, e, g, i, k and m). Upon lysis of the cells by French pressure in the presence of Triton X-100, all of the fusion proteins (except for the N-domain) were found to be present in the high speed supernatant fraction. This is in contrast to many proteins expressed in E. coli, which were recovered in insoluble inclusion bodies. The solubility of the fusion proteins supported the suggestion that they might be properly folded within E. coli and therefore might be able to bind Ca^{2+}. The N-domain was somewhat less soluble than the other regions of calreticulin but approximately 20% of this fusion protein was still found in the supernatant fraction (see Fig. 3-6A).

The GST-fusion proteins were purified by one step affinity chromatography on Glutathione-Sepharose (Fig. 3-5B, lanes d, f, h, j, l and n). Figures 3-5C and D show that the C-domain (lane f), P-domain (lane h) and P+C-domain (lane l) reacted with anti-calreticulin antisera and all bound $^{45}Ca^{2+}$. In contrast, the N-domain, (lane j) reacted only weakly with the goat anti-calreticulin antibodies and did not bind detectable amounts of $^{45}Ca^{2+}$ (Fig. 3-5C and D, respectively).

Purification of Calreticulin domains minus GST

All GST-calreticulin fusion proteins were digested with Factor Xa (as described in "Chapter Two: Experimental Procedures") to remove GST in order to obtain calreticulin domains (free of GST) for further biochemical analysis.

Purification of the N-domain The GST-N-domain fusion protein was expressed in E. coli. and the cells lysed to release expressed proteins. The soluble fraction (Fig. 3-6A, lane d) had a considerably less amount of the GST-N-domain fusion protein compared to the pellet sample (Fig. 3-6A, lane e). The N-domain contains a large number of relatively hydrophobic amino acids. The hydrophobicity of the GST-N-domain fusion protein made it insoluble and hence it was not surprising that very little sample was present in the supernatant after solubilization of the overexpressing E. coli cells (Fig. 3-6A, lane d). An SDS-PAGE analysis of the GST-N-domain fusion protein extracted from the insoluble pellet is shown in Fig. 3-6A. The GST-N-domain fusion protein was extracted from the insoluble pellet using the procedure of Lin and Cheng (1991) and it involved the extraction of the GST-N-domain out of the pellet, denaturation of the sample, followed by renaturation overnight to obtain a soluble form of the GST-N-domain sample. Approximately 40% of the GST-N-domain found in the pellet was extracted by this method of Lin and Cheng (1991) (Fig. 3-6A, lane l) and was further purified by Glutathione-Sepharose affinity chromatography. In all subsequent experiments, only the soluble fraction of the GST-N-domain was used.

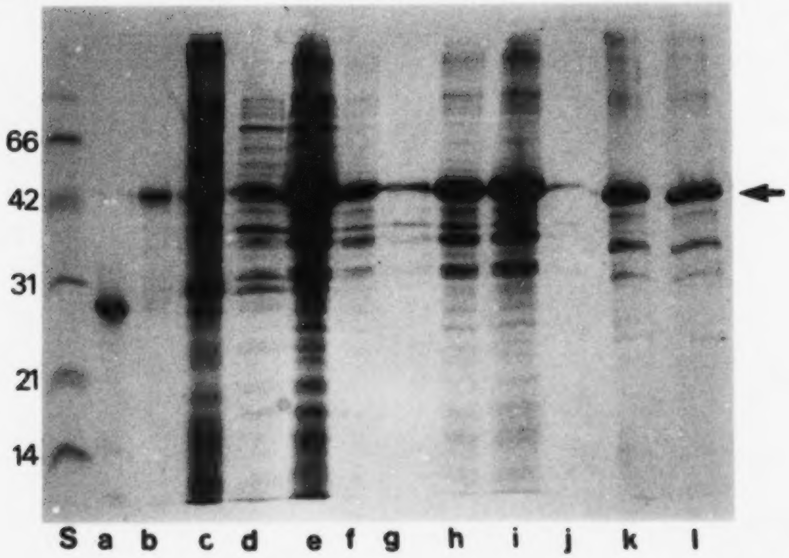
In order to obtain the N-domain fragment of calreticulin that is

Figure 3-6: SDS-PAGE of the N-domain of calreticulin

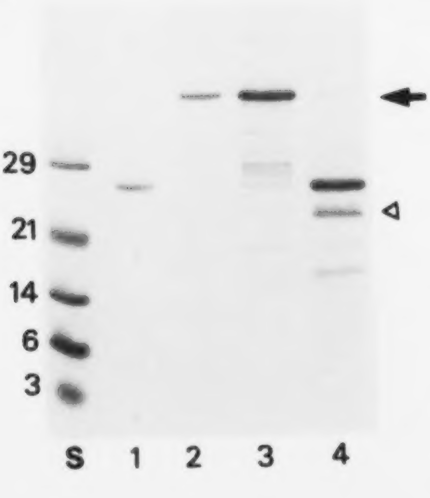
(A) N-domain fusion protein was expressed in E. coli., lysed to release expressed proteins, and the samples were analyzed by SDS-PAGE (12.5 % gel). The soluble fraction (lane d) had a considerably lower amount of the GST-N-domain fusion protein compared to the pellet sample (lane e). The GST-N-domain present in the insoluble pellet was extracted as outlined in "Chapter Two: Experimental Procedures". Lane a, Glutathione-Sepharose purified recombinant GST; lane b, Glutathione-Sepharose purified recombinant GST-N-domain fusion protein; lane c, uninduced control E. coli,. cells, soluble fraction; lane d, induced GST-N-domain E. coli. cells, soluble fraction (containing a small amount of the GST-N-domain); lane e, induced GST-N-domain E. coli. cells, insoluble pellet fraction (containing majority of the GST-N-domain sample); lane f, **stage 1** - pellet after hypotonic shock; lane g, **stage 2** - resuspended pellet after hypotonic shock; lane h, **stage 3** - pellet after RNase T1, DNase 1 digestion and sonication; lane i, **stage 4** - pellet after buffer W wash; lane j, **stage 5** - pellet after denaturation and sonication; lane k, **stage 6** - supernatant after denaturation and sonication; lane l, **stage 7** - extracted GST-N-domain. The position of the GST-N-domain is indicated by the arrow. S, Bio-Rad low molecular weight standards as indicated.

(B) Extracted GST-N-domain was digested with Factor Xa, separated on SDS-PAGE (15 % gel), and then stained with Coomassie Blue. Lane 1, Purified recombinant GST; lanes 2 and 3, Glutathione-Sepharose purified recombinant GST-N-domain fusion protein; lane 4, factor Xa cleaved GST-N-domain fusion protein (after 16 hours). The position of the isolated N-domain is indicated by the open triangle. The position of the GST-N-domain is indicated by the arrow. S, Bio-Rad low molecular weight standards as indicated.

A


66
42
31
21
14
S
a
b
c
d
e
f
g
h
i
j
k
l

B


29
21
14
6
3
S
1
2
3
4

free of GST, the GST-N-domain sample (Figure 3-6B, lanes 2 and 3) was first digested with Factor Xa for 8 - 10 hours (Figure 3-6B, lane 4) and then applied onto a Glutathione-Sepharose affinity column to remove GST. Two bands appeared corresponding to GST (26 kDa) and the N-domain of calreticulin (23.7 kDa) (indicated by the open triangle). This is close to the predicted molecular weight of 21 500 based on the amino acid sequence. Anion exchange and hydrophobic interaction chromatographies were used to remove the contaminating non-N-domain bands in Fig. 3-6B, lane 4. Both chromatographies, however, failed to separate the contaminating bands (mainly GST) from the N-domain band. In order to obtain this protein band in pure form, the N-domain protein band was cut out from the gel and electroeluted. Thirty to fifty micrograms of the N-domain was obtained by the electroelution procedure. Identity of the protein band was confirmed by NH_2-terminal amino acid sequence analysis which revealed a sequence of G-N-S-E-P-V-V. This corresponded to the multiple cloning site amino acids (underlined sequence) and to the NH_2-terminus of mature, full length rabbit skeletal calreticulin. The goat anti-calreticulin antibody did not react to this domain of calreticulin (Fig. 3-5B, lane j) and we have not been able to generate a specific antibodies against the N-domain. This region of calreticulin has the most conserved amino acid sequence in all calreticulin forms obtained to date (see Fig. 1-3). This may be a reason for its low immunogenic activity and hence our inability to obtain an N-domain specific antibody.

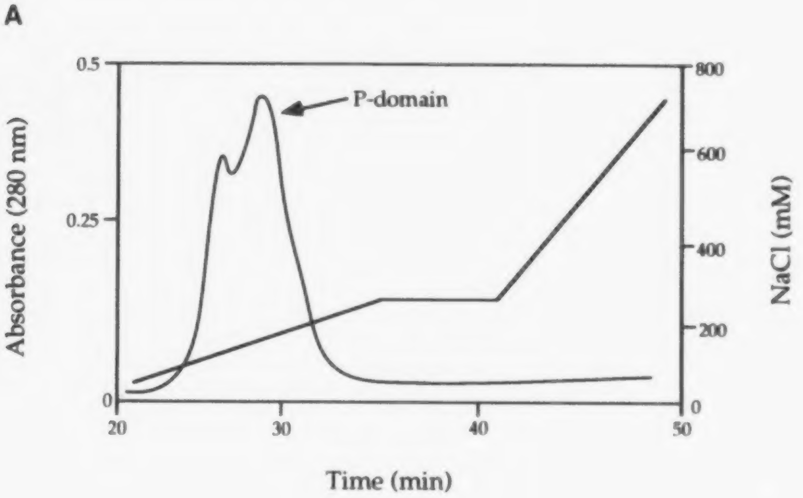
Purification of the P-domain The P-domain was purified by the Glutathione-Sepharose affinity chromatography (Figure 3-5B, lane h) followed by digestion with Factor Xa for 16 hours (Fig. 3-7B, lane c). Two protein bands were obtained after Factor Xa digestion - a 26 kDa GST band (indicated by the open triangle) and a 21 kDa P-domain band (indicated by the arrow). This mixture containing both GST and the P-domain was applied onto a Glutathione-Sepharose affinity column to remove GST (which binds to the column) and the flowthrough from the Glutathione-Sepharose affinity column (Fig. 3-7B, lane c), containing the P-domain was applied onto a Mono Q FPLC anion exchange column to purify the P-domain sample. A step gradient was used for the elution of the P-domain off the Mono Q column. The P-domain eluted at 159 mM NaCl (Fig. 3-7A and Fig. 3-7C, lane 29, indicated by the arrow) in a pure form. The GST band in the P-domain sample appeared in the flowthrough fractions (lanes 1 to 20) and was also eluted in fractions 27 and 28. This GST band was well separated from the P-domain fragment (Fig. 3-7C, compare lanes 27 - 29). Calreticulin (Fig. 3-8C, lane a) and the 21 kDa P-domain band (Fig. 3-8C, lane e) were shown to react with the goat anti-P-domain antibody, and no reaction was observed for GST, bovine serum albumin, and cardiac calsequestrin (Fig. 3-8C, lanes b, c, and d, respectively) indicating the specificity of this antibody. NH_2-terminal amino acid analysis of the 21 kDa P-domain (Fig. 3-7C, lane 29) revealed the presence of two protein bands (Fig. 3-8A, lane 5):

PM233U - ^{191}IKDPDASKPEDWD............PDYKGTWI^{273} (upper band of doublet)

PM233L - ^{196}ASKPEDWDERAIKI...........PDYKGTWI^{273} (lower band of doublet)

Figure 3-7: Mono Q FPLC chromatography of Factor Xa cleaved GST-P-domain

GST-P-domain fusion protein was purified by Glutathione-Sepharose affinity chromatography, cleaved with factor Xa (16 hours), passed over a second Glutathione Sepharose column (to remove GST) and purified by Mono Q FPLC chromatography as described under "Chapter Two: Experimental Procedures". The protein was applied to an FPLC Mono Q 5/5 anion exchange column followed by elution with a step NaCl gradient (50 - 225 mM NaCl; and 225 - 750 mM NaCl). One milliliter fractions were collected and absorption at 280 nm and NaCl concentrations were monitored. SDS-PAGE (12.5 % gel) of the starting material (lane c), flowthrough fractions (B), and of eluted peak fractions (C) are shown. Two milliliter fractions were collected and analyzed on SDS-PAGE (fraction numbers are indicated). The position of the P-domain protein peak is indicated by the arrow and GST by the open triangle. S, Bio-Rad low molecular weight standards as indicated.


A
0.5
0.25
0
Absorbance (280 nm)
P-domain
800
600
400
200
0
NaCl (mM)
20
30
40
50
Time (min)

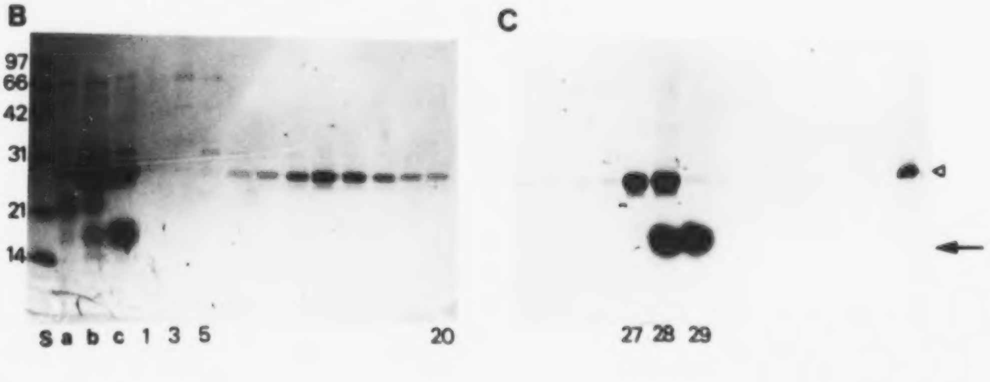
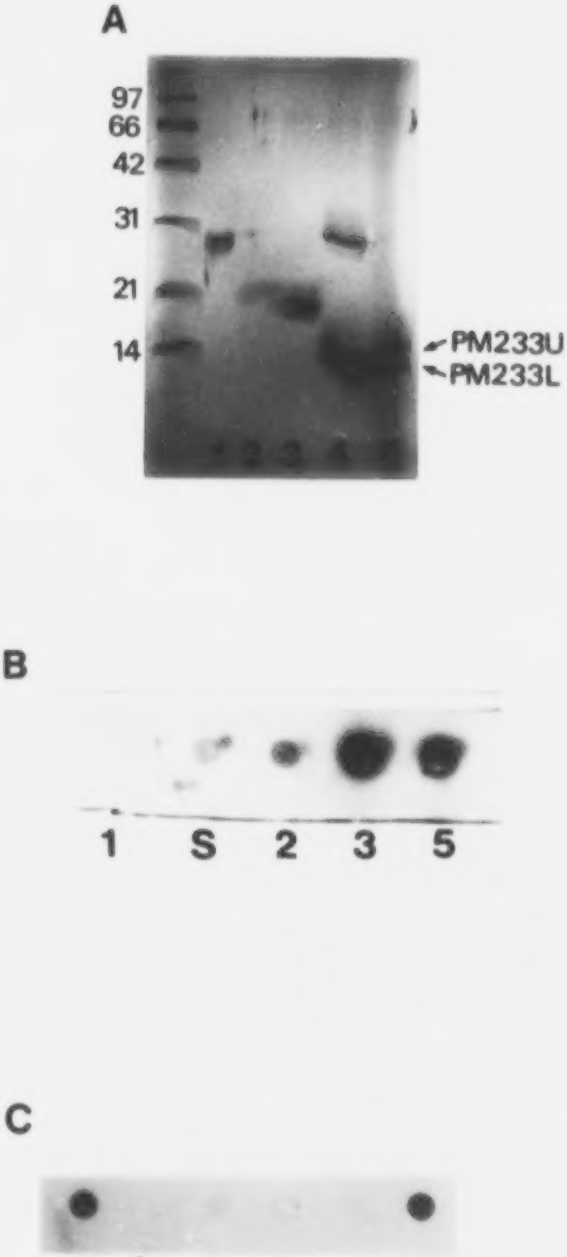

B
97
66
42
31
21
14
S a b c 1 3 5
20
C
27 28 29

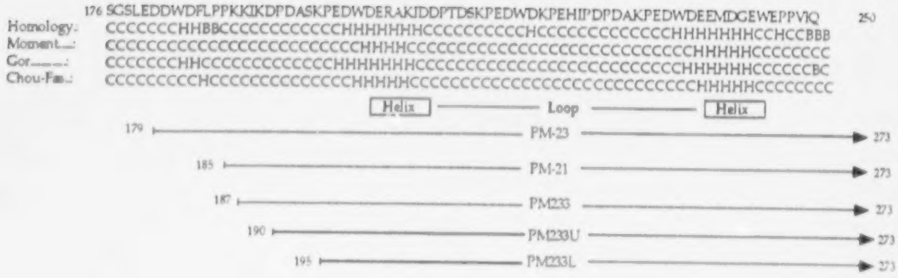
Figure 3-8: Characterization of truncated P-domain fragments

Truncated P-domain fragments (PM-21, PM-23 and PM233) were obtained via Factor Xa proteolytic cleavage and were separated by SDS-PAGE (12.5 % gel), transferred electrophoretically or dot blotted to nitrocellulose membranes and incubated with $^{45}Ca^{2+}$ or with anti-calreticulin antibody as described under "Chapter Two: Experimental Procedures.". A, Coomassie Blue staining; B, autoradiography of samples in A after dot blotting onto nitrocellulose membrane and incubation with $^{45}Ca^{2+}$; C, immunostaining with goat anti-P-domain antibody; . **For (A) and (B):** Lane 1, GST; lane 2, PM-21 fragment ; lane 3, PM-23 fragment; lane 4, fraction 28 of Fig. 3-7C; lane 5, fraction 29 of Fig. 3-7C; lane S, dog pancreatic calreticulin. In **(A)** see Fig. 3-9 for sequence of PM233U and PM233L. **For (C):** lane a, dog pancreatic calreticulin; lane b, GST; lane c, bovine serum albumin; lane d, cardiac calsequestrin; lane e, PM233. All proteins are Mono Q FPLC purified samples. The positions of molecular weight marker proteins are indicated.


A
97
66
42
31
21
14
PM233U
PM233L
1 2 3 4 5
B
1
S
2
3
5
C
a
b
c
d
e

Hence, it appeared that the lower band differed from the upper band by six amino acids. Digestion of a different preparation of the GST-P-domain protein with Factor Xa produced two more proteolytic fragments (PM-21 and PM-23) that corresponding to truncated regions within the P-domain (Fig. 3-8A, lanes 2 and 3, respectively and Fig. 3-9A). All of these fragments bound Ca^{2+} as determined by the $^{45}Ca^{2+}$ overlay technique (Fig. 3-8B). We concluded that Factor Xa cleaved the GST-P-domain at sites other than its recognition amino acid sequence of I-E-G-R. The amino acid sequences of the different fragments of the P-domain are shown in Fig. 3-9A and some of their characteristics are shown in Fig. 3-9B. The P-domain proteolytic fragments obtained encompass a region of the protein that was found to contain the high affinity Ca^{2+} binding site (see in Chapter Four: Identification of the calcium binding sites within calreticulin). As mentioned earlier, calreticulin does not contain an EF-hand consensus amino acid sequence that is present in high affinity Ca^{2+} binding proteins such as calmodulin and troponin C (Kretsinger, 1988). Calreticulin, however, was predicted to have the secondary structural features associated with the EF-hand consensus amino acid sequence - that is the helix-loop-helix secondary structure (Fig. 3-9A). This type of structure provides the necessary interactions for the high affinity binding of Ca^{2+}. All of the P-domain proteolytic fragments produced contain this putative helix-loop-helix motif in their amino acid sequences and this may explain their abilities to bind Ca^{2+} (Fig. 3-8B). These results provided the impetus for the construction of the truncated GST-P-domain fusion proteins in order to determine the possible localization of the high affinity Ca^{2+} binding site within calreticulin (see in Chapter

A


176 SGSLEDDWDFLPPKKIKDPDASKPEDWDERAKIDDPTDSKPEDWDKPEHIPDPDAKPEDWDEEMDGEWEPPVIQ 250
Homology: CCCCCCCHHBBCCCCCCCCCCCCHHHHHHHCCCCCCCCCCHCCCCCCCCCCCCCHHHHHHHCCHCCBBB
Moment: CCCCCCCCCCCCCCCCCCCCCCCCCCHHHHCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCHHHHHCCCCCCCC
Gor: CCCCCCCHHCCCCCCCCCCCCCCHHHHHHHCCCCCCCCCCCCCCCCCCCCCCCCCCCCCHHHHHHCCCCCCBC
Chou-Fas: CCCCCCCCCHCCCCCCCCCCCCCCHHHHHCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCHHHHHCCCCCCCC
Helix
Loop
Helix
179
PM-23
273
185
PM-21
273
187
PM233
273
190
PM233U
273
195
PM233L
273

B

	Mr (kDa)		
	cDNA	SDS-PAGE	No. of amino acids
PM-21	10.9	20.6	95
PM-23	10.1	19.3	87
PM233U	9.5	16.1	82
PM233L	8.8	15.7	76

Figure 3-9: Amino acid sequence and molecular masses of truncated P-domain fragments

This figure summaries the amino acid sequence data of the truncated P-domain fragments (A) and provides molecular weight information (B). The secondary structure algorithms Homology, Moment, Gor, and Chou-Fas were carried by David Wishart of the University of Alberta PENCE group using the Seqsee program (Wishart *et al.*, 1994).

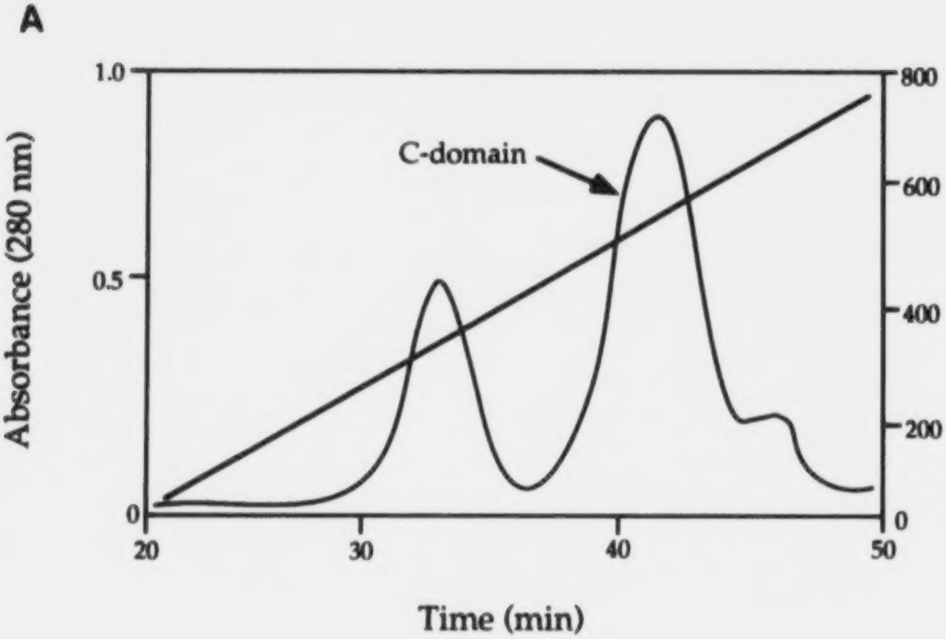
Four: Identification of the calcium binding sites within calreticulin).

Purification of the C-domain The C-domain was expressed as a GST fusion protein and purified by Glutathione-Sepharose affinity chromatography (Figure 3-5B, lane f). The GST-C-domain was digested with Factor Xa for 16 hours (Fig. 3-12A, lane c). SDS-PAGE analysis of the digested sample revealed two protein bands - a 26 kDa GST band and a 23 kDa C-domain protein band (Fig. 3-10B, lanes 37 - 39, indicated by the arrow). This mixture of GST and the C-domain was applied onto a Mono Q FPLC column and a linear gradient was used for the elution of the C-domain from the Mono Q column. The C-domain eluted in three fractions at approximately 560 mM NaCl (Fig. 3-10A and Fig. 3-10B, lanes 37 - 39). A 18.8 kDa protein band (Fig. 3-10B, lane 31) eluted at approximately 300 mM NaCl and both this protein band and the 23 kDa C-domain protein band reacted with the goat anti-calreticulin antibody and bound Ca^{2+} as measured by the $^{45}Ca^{2+}$ overlay technique (Fig. 3-12B, lane e). The 18.8 kDa protein band was likely a degraded product of the 23 kDa C-domain.

Purification of the N+P-domain The N+P-domain was expressed as a GST fusion protein and purified by Glutathione-Sepharose affinity chromatography (Figure 3-5B, lane n), followed by digestion with Factor Xa for 6 - 16 hours (Fig. 3-12A, lane e and f, respectively). As a result of the digestion with Factor Xa three protein bands were obtained - a 36 kDa, a 31 kDa, and a 26 kDa band. The 26 kDa protein band corresponded to GST and NH_2-sequence analysis revealed that the 36 kDa protein band

Figure 3-10: Mono Q FPLC chromatography of Factor Xa cleavage Glutathione-Sepharose purified recombinant GST-C-domain fusion protein

The GST-C-domain fusion protein was purified by Glutathione-Sepharose affinity chromatography, cleaved with factor Xa (16 hours), passed over a second Glutathione Sepharose column (to remove GST) and purified by Mono Q FPLC chromatography as described under "Chapter Two: Experimental Procedures". The protein was applied to an FPLC Mono Q 5/5 anion exchange column followed by elution with a linear (50-750 mM) NaCl gradient. One milliliter fractions were collected and absorption at 280 nm and NaCl concentrations were monitored. SDS-PAGE (12.5 % gel) of the starting material (lane a), and of peak fractions (remaining lanes) are shown. Lane b, purified GST. The position of the C-domain protein peak is indicated by the arrow. The 52 kDa protein band in lane 40 corresponds to the undigested GST-C-domain fusion protein. The fraction numbers are indicated and correspond to (A). The positions of molecular weight marker proteins are indicated (lane S).


A
1.0
0.5
0
Absorbance (280 nm)
800
600
400
200
0
C-domain
20
30
40
50
Time (min)

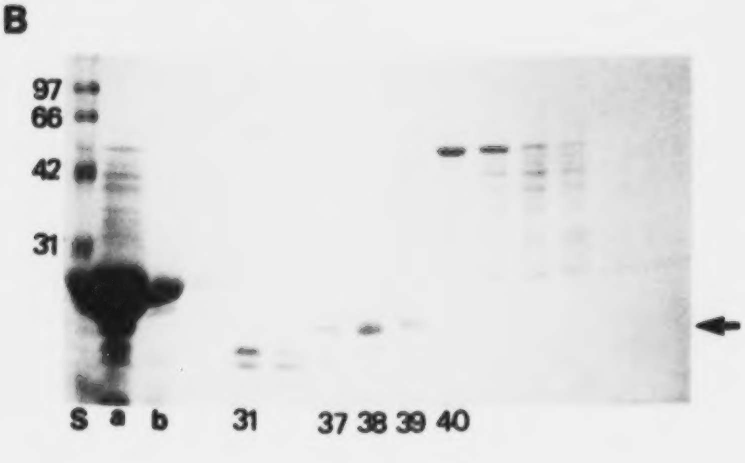
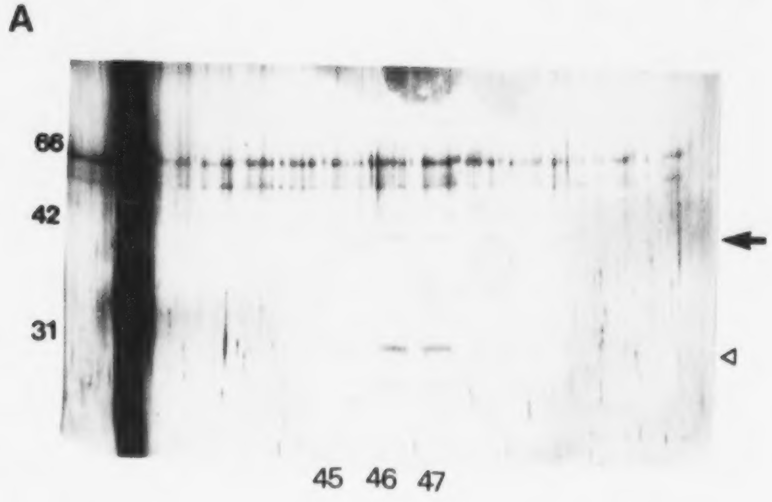
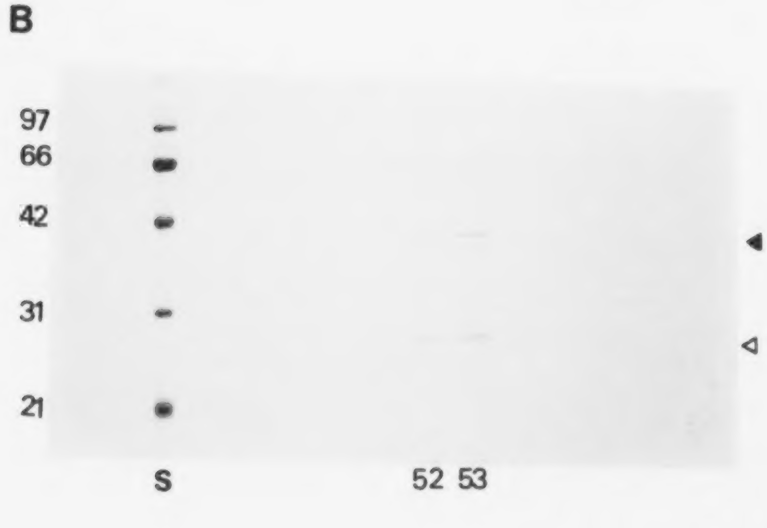

B
97
66
42
31
S
a
b
31
37
38
39
40

Figure 3-11: SDS-PAGE of the N+P-domain and P+C-domain of calreticulin

The GST-N+P-domain (A) and the GST-P+C-domain (B) fusion protein were purified by Glutathione-Sepharose affinity chromatography, cleaved with factor Xa, passed over a second Glutathione Sepharose column (to remove GST) and purified by Mono Q FPLC chromatography as described under "Chapter Two: Experimental Procedures". The protein was applied to an FPLC Mono Q 5/5 anion exchange column followed by elution with a linear (50-750 mM) NaCl gradient. One milliliter fractions were collected and absorption at 280 nm and NaCl concentrations were monitored. SDS-PAGE (12.5 % gel) of the eluted fractions are shown and fraction numbers indicated (this gel was silver stained). The position of the N+P-domain protein peak is indicated by the arrow and the position of the P+C-domain protein peak is indicated by the closed triangle. GST is indicated by the open triangle. The position of molecular weight marker proteins is indicated in S. In A, two diffuse protein-like bands (66 kDa and 58 kDa) appear in all the lanes. This is due to the silver staining reagent and does not reflect eluted proteins from the column.


A
66
42
31
45
46
47


B
97
66
42
31
21
S
52
53

corresponded to full length N+P-domain, whereas the 31 kDa protein band was missing the first 56 NH_2-terminal residues of calreticulin. This will be referred to throughout this thesis as the truncated N+P-domain. Separation of these three protein bands was achieved using Mono Q FPLC chromatography and a linear gradient was used for the elution of the N+P-domain off the Mono Q column (Fig. 3-11A). Two protein bands were obtained - a 26 kDa GST band (open triangle) and a 36 kDa N+P-domain protein band (arrow). The N+P-domain eluted in three fractions at 465 mM NaCl (Fig. 3-11A, lanes 45 - 47) and appeared to have coeluted with a 26 kDa GST band. Surprisingly, the 31 kDa protein band did not bind to the column and appeared in the flowthrough. The GST band was then removed from the sample in lanes 45 - 47 by Glutathione-Sepharose affinity column and the flowthrough contained pure N+P domain. The identity of the N+P-domain was confirmed by its reactivity with the goat anti-calreticulin antibody and then by the $^{45}Ca^{2+}$ overlay technique (Fig. 3-12B, lane g).

Purification of the P+C-domain The P+C-domain was expressed as a GST fusion protein and purified by Glutathione-Sepharose affinity chromatography (Figure 3-5B, lane l), followed by digestion with Factor Xa for 16 hours (Fig. 3-12A, lane h). SDS-PAGE analysis of the Factor Xa digested sample revealed three bands - a 36 kDa and a 31 kDa goat anti-calreticulin immunoreactive bands; and a 26 kDa GST band (Fig. 3-12A, lane h, indicated by the arrow). Further purification of the P+C-domain was achieved on Mono Q FPLC column. A linear gradient was used for the elution of the P+C-domain from the Mono Q column (Fig. 3-11B).

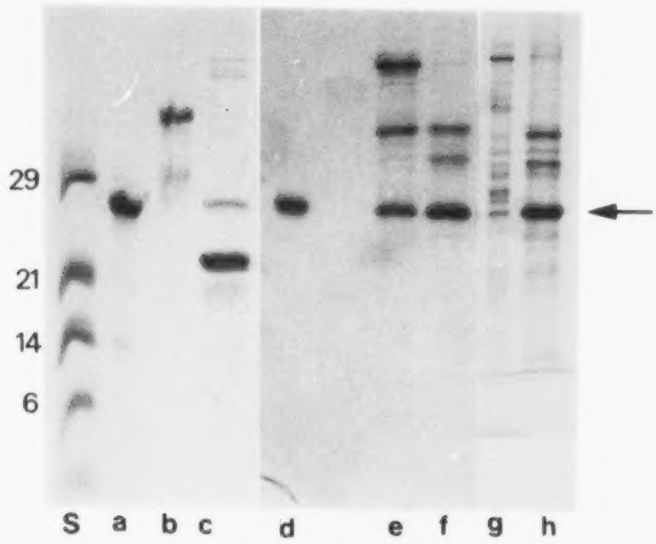
Figure 3-12: SDS-PAGE of GST-free calreticulin domains

Calreticulin domains were separated by SDS-PAGE (15 % gel) and Coomassie blue stained (A) or transferred electrophoretically to nitrocellulose membranes and incubated with $^{45}Ca^{2+}$ (B) as described under "Chapter Two: Experimental Procedures."

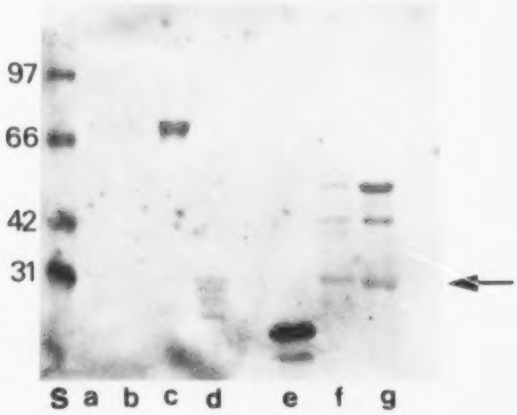
(A) Lanes a and d, Glutathione S-transferase purified GST; lane b, Glutathione S-transferase purified GST-C-domain fusion protein; lane c, Factor Xa cleaved GST-C-domain fusion protein (16 hours); lanes e and f, Factor Xa cleaved GST-N+P-domain fusion protein (6 hours and 16 hours respectively); lane g, Glutathione S-transferase purified GST-P+C-domain fusion protein; lane h, Factor Xa cleaved GST-P+C-domain fusion protein (16 hours). Arrow indicates the position of GST. S, Mandel low molecular weight standards.

(B) Lane a, purified GST; lane b, bovine serum albumin; lane c, dog pancreatic calreticulin; lane d, N-domain; lane e, C-domain.; lane f, P+C-domain; lane g, N+P-domain. S, Bio-Rad low molecular weight standards as indicated. Arrow indicates the position of GST. The appearance of the Mr standards in B is due to the light emission from the dye bound to the standards proteins.

A


29
21
14
6
S
a
b
c
d
e
f
g
h

B


97
66
42
31
S
a
b
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d
e
f
g

The P+C-domain eluted in three fractions at 530 mM NaCl (Fig. 3-11B, lanes 52 - 53) together with a 26 kDa GST protein band. The 31 kDa protein band did not bind to the Mono Q column and was found in the column flowthrough. Glutathione-Sepharose affinity chromatography was used to remove GST from the sample in lanes 52 - 53 and pure P+C domain was found in the Glutathione-Sepharose affinity column flowthrough. The identity of the P+C-domain was then confirmed by immunoreactivity with goat anti-calreticulin antibody and then by the $^{45}Ca^{2+}$ overlay technique (Fig. 3-12B, lane f).

DISCUSSION

In this chapter, we have described methods for the purification of recombinant and native calreticulin in quantities sufficient to allow careful biochemical, functional and ultrastructural characterization. Native calreticulin was purified by selective ammonium sulfate precipitation followed by DEAE-Sephadex and hydroxylapatite chromatography. Application of the GST-fusion protein system allowed us to express large quantities of recombinant calreticulin and calreticulin domains, which were subsequently purified by Glutathione-Sepharose and Mono Q FPLC chromatographies. Both native and recombinant calreticulin were of high purity as determined by SDS-PAGE, their NH_2-terminal amino acid sequence analysis, their reverse phase HPLC chromatography profiles, their reactivity with anti-calreticulin antibodies, and finally their Ca^{2+} binding properties. All of the chromatography matrices used in these studies are commercially available, making these methods readily accessible to all laboratories interested in studying the structure and function of calreticulin.

The procedure for the purification of native calreticulin developed in our laboratory introduces a number of major improvements over the methods used earlier (Ostwald and MacLennan, 1974; Michalak *et al.*, 1990; and MacLennan, 1984). MacLennan's group developed a procedure for the purification of calreticulin (earlier known as "the high affinity Ca^{2+}-binding protein") from isolated skeletal muscle SR vesicles. The protein was extracted from the lumen of sarcoplasmic reticulum vesicles with a low concentration of detergent, in the presence of 1 M KCl, followed by DEAE cellulose and hydroxylapatite chromatography. This procedure does not involve selective precipitation of calreticulin with ammonium sulfate. Waisman's group (Waisman, 1985) used liver homogenates for the purification of calreticulin ("calregulin"). Their procedure, however, requires freeze-thawing of the liver in order to release membrane-bound calreticulin. The procedure developed in our laboratory completely eliminates the need for lengthy preparation of membrane fractions and results in high yield and purity. Moreover, the method can be used for the purification of calreticulin from any tissue and/or any cells in culture.

II. Biochemical characterization of calreticulin and calreticulin domains

INTRODUCTION

Calreticulin is a multifunctional protein, which is implicated to be involved in a variety of cellular functions (Michalak *et al.*, 1992). Post-translational modifications of the protein may be responsible for these diverse functions. For example, species-specific variations are seen in post-translational modification features of calreticulin such as phosphorylation and glycosylation (for review see Michalak *et al.*, 1992). In this section, an analysis of the anomalous migration of the protein in SDS-PAGE was investigated. In addition, analysis of the disulfide bond content, phosphorylation, glycosylation, and RNA binding ability of canine pancreatic calreticulin were examined. These findings may facilitate an understanding of the post-translational processing of canine pancreatic calreticulin and how it may be related to the protein's function in varied locations.

RESULTS AND DISCUSSION

The anomalous migration of calreticulin

The calculated isoelectric point of calreticulin is 4.14 (Fliegel *et al.*, 1989b; McCauliffe *et al.*, 1990a) and the value measured for the native protein is 4.65-4.67 (Waisman *et al.*, 1985; McCauliffe *et al.*, 1990a). Based on the amino acid sequence deduced from its cDNA, the molecular mass of calreticulin was estimated to be 46 567 Da (Fliegel *et al.*, 1989b, Smith and Koch, 1989). However, when analyzed by SDS-PAGE (Laemmli system) calreticulin migrates with an apparent molecular weight of 55 000 - 60 000 (Waisman *et al.*, 1985; McCauliffe *et al.*, 1990a; Milner *et al.*, 1991). The molecular weight of calreticulin estimated by SDS-PAGE at neutral pH is 55 000 (Ostwald and MacLennan, 1974; Michalak *et al.*, 1980) which is similar to the value determined by sedimentation equilibrium, also at

Table 3-2: Mobility of calreticulin and calreticulin domains on SDS-PAGE

CRT/CRT domain	cDNA M_r	SDS-PAGE M_r	% Difference
Tissue CRT	46 567	55 814	20
RCRT	47 245	61 099	29
GST-N	42 651	42 957	0.7
GST-P	42 830	42 651	0.4
GST-C	37 266	39 574	6.19
GST-N+P	59 687	61 567	3.1
GST-P+C	57 975	63 257	9.11
GST-CRT	73 446	79 588	8.4
N	21 515	23 700	10
P	15 951	22 583	42
C	15 145	22 334	48
N+P	33 350	37 500	12
P+C	30 779	36 805	20
GST	26 879	26 919	0.1

Abbreviations:

CRT - calreticulin; RCRT - recombinant CRT; N - N-domain of CRT; P - P-domain of CRT; C - C-domain of CRT; N+P - N+P-domain of CRT; PC - P+C-domain of CRT; GST-CRT - fusion protein containing the entire amino acid sequence of CRT. SD ± 1200 Da (n = 10).
"% difference" denotes difference between predicted and actual mobility on SDS-PAGE.

neutral pH. Table 3-2 lists the molecular masses of calreticulin and the various fusion proteins based on amino acid sequence deduced from the rabbit skeletal cDNA clone of Fliegel *et al.* (1989b) and molecular mass on SDS-PAGE. Both canine pancreatic calreticulin and recombinant calreticulin ran abberantly on SDS-PAGE, having molecular weights of 20% and 29% higher than predicted from their cDNA. The GST-N-domain (GST-N) and GST-P-domain (GST-P) fusion proteins ran approximately at their calculated molecular weights, whereas the GST-C-domain (GST-C) ran at 6.19% higher than its calculated molecular weight. GST-N+P-domain (GST-NP) and GST-P+C-domain (GST-PC) fusion proteins also ran at 3.1% and 9.11% higher than they should. Similarly, the full length calreticulin fusion protein (GST-CRT) ran at 8.4 % higher than it should. We concluded, therefore, that it is the C-domain of calreticulin that may be responsible for the aberrant migration of calreticulin on SDS-PAGE. When GST was removed from these samples, the N-domain, P-domain and C-domain ran at 10%, 42%, and 48% higher than calculated. The N+P-domain and the P+C domain ran at 12% and 20% higher than their predicted molecular weights based on their amino acid composition. Again, these calculations reveal that the aberrant migration of calreticulin on SDS-PAGE may be predominantly caused by the C-domain and to lesser extent the P-domain. Both of these domains are highly acidic and contain a high percentage of negatively charged residues. The discrepancies between predicted and observed mobilities on SDS-PAGE have been reported for several other proteins, including calsequestrin, the "Ca^{2+}-storage" protein of muscle SR (MacLennan *et al.*, 1983). Like calsequestrin, calreticulin has a highly charged carboxyl-terminal region. This acidity is thought to be responsible for the aberrant migration in gels of both proteins.

Oligomeric structure of calreticulin

Sedimentation equilibrium is useful in the determination of molecular weight of a sample and also for the detection of monomer-dimer associations. A plot of the natural log of the concentration of the protein sample (recombinant calreticulin) versus the distance traveled was linear indicating homogeneity of the protein sample. Analysis of the plot revealed that calreticulin had an average mass of 54 160 Da and appeared to form a monomeric species. Dimeric or higher polymers would have produced molecular weights of 108 320, 162 480, and higher and a curvature in the plot of lny versus r^2. Recent high resolution STEM (scanning tunneling electron microscopy) analysis has suggested that calreticulin may aggregate to form dimers and/or tetramers (Baksh, S., Andrews, D., and Michalak, M., unpublished results). We have never observed high immunoreactive calreticulin bands using SDS-PAGE. The STEM analysis was carried out at a protein concentration of 0.5 - 1 mg/ml. It is possible that at high protein concentrations could induce polymeric state(s). STEM analysis at very dilute protein concentrations would indicate the true oligomeric state of calreticulin.

Sedimentation velocity yielded a sedimentation coefficient ($s_{20,w}$) of 2.81 for the native form of calreticulin. No change in this value was observed when Ca^{2+} was added. Calreticulin undergoes very little changes in conformation upon binding of its ions or in its interaction with proteins.

Disulfide bond analysis

Calreticulin has three cysteine residues within the highly structured N-domain. It was shown by Matsuoka *et al.*, (1994) that two of these cysteines are involved in an intramolecular disulfide bond - Cys-120 and Cys-146. These cysteine residues are conserved throughout all calreticulin proteins sequenced to date (see Fig. 1-3). It is therefore possible that all calreticulin species may have this intramolecular disulfide bond within the N-domain to stabilize the tertiary organization of the protein. Titration of purified canine pancreatic calreticulin with Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid) or DTNB) revealed 0.95 moles of free thiol group / mole of calreticulin. Similarly, rabbit liver calreticulin produced 0.86 moles free thiol group / mole of calreticulin. Since all three cysteines are conserved in all known calreticulin species thus far, we assumed that canine pancreatic calreticulin would also have this one intramolecular 25-amino acid disulfide linked loop between Cys-120 and Cys-146.

Stains-All Staining

Acidic proteins which bind Ca^{2+}, such as calreticulin (Fliegel *et al.*, 1989b; Krause *et al.*, 1990) and calsequestrin (Campbell *et al.*, 1983), stain blue with Stains-All, whereas other proteins stain pink (Campbell *et al.*, 1983). Stains-All is a cationic carbocyanine dye that interacts with different macromolecules and causes changes in the absorption spectrum of the dye, resulting in the different colored dye-protein complexes (Campbell *et al.*, 1983). Figure 3-13A showed Stains-All staining of the same protein samples in Fig. 3-3D. Native calreticulin (Fig. 3-13A, lane 1),

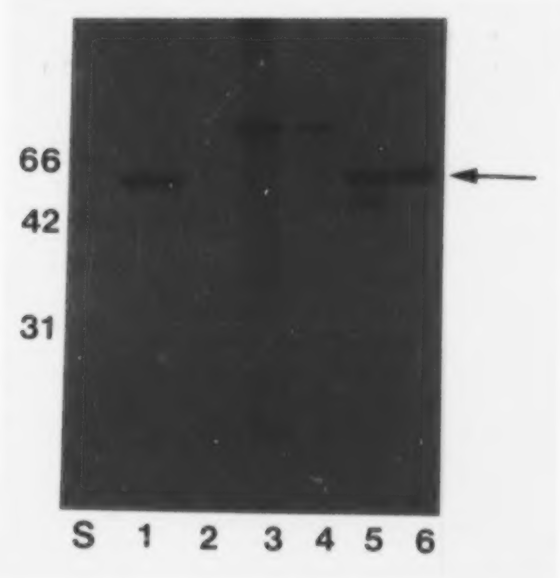
Figure 3-13: Stains-All staining of calreticulin and calreticulin domains

Proteins were separated by SDS-PAGE and stained with the cationic carbocyanine dye Stains-All, as described by Campbell *et al.* (1983) and "Chapter Two: Experimental Procedures".

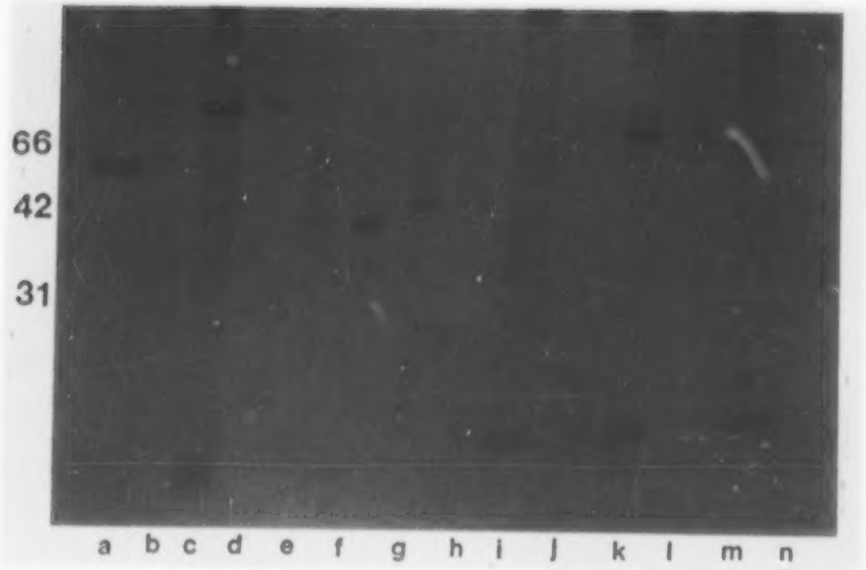
(A) Native and recombinant calreticulin. Protein samples were the same as in Figure 3-3D. The arrow indicates the position of calreticulin and the position of the molecular weight marker proteins are indicated.

(B) Calreticulin domains. Protein samples were the same as in Figure 3-5B. The position of the molecular weight marker proteins are indicated.

A


66
42
31
S 1 2 3 4 5 6

B


66
42
31
a b c d e f g h i j k l m n

GST-calreticulin fusion protein (Fig. 3-13A, lane 3) and recombinant calreticulin (Fig. 3-13A, lane 6) all stained blue with Stains-All, further supporting Ca^{2+} binding to both native and recombinant calreticulin samples. All other proteins, including recombinant GST, stained pink (Fig. 3-13A, lane 2. Figure 3-13B showed Stains-All staining of the GST fusion proteins containing calreticulin domains. The full length calreticulin fusion protein (lane d), the GST-C-domain (lane f), the GST-P-domain (lane h), the GST-P+C-domain (lane l), and the GST-N+P-domain (lane n) also stained blue with Stains-All further confirming their involvement in Ca^{2+} binding to calreticulin. The GST-N-domain (lane j) and GST (lane b) did not stain blue and therefore may not be involved in Ca^{2+} binding. This provided preliminary evidence for the localization of the Ca^{2+} binding regions to the P-domain and to the C-domain of calreticulin.

Carbohydrate analysis

The importance of glycosylation in calreticulin is currently unclear. Whilst the amino acid sequence of the protein contains one potential glycosylation site (Asn-326: Asn-Xaa-Ser/Thr), it is only utilized in some species of calreticulin. Enzymatic analysis of the carbohydrate (CHO) content of calreticulin was carried out using various endoglycosidases. Endoglycosidases cleave off CHO moieties from proteins and allows the protein to move faster in SDS-PAGE because of the lower mass it now assumes. Treatment of canine pancreatic calreticulin or recombinant calreticulin with endoglycosidase H, endoglycosidase F, endoglycosidase D, and glycopeptidase F did not produce any shift in their mobility in

SDS-PAGE indicating that canine calreticulin may not contain sugar residues that are cleaved by these enymes.

Chemical analysis of the possible CHO content of calreticulin was measured by the phenol/H_2SO_4 method of Dubois *et al.* (1956). This method was used to determine the total CHO content of a sample. Using glucose as a standard, we obtained a total content of 185 μg CHO / mg of canine cardiac calsequestrin or 47 μmoles CHO / μmole protein. Calreticulin gave us a value of 43 μg CHO / mg of protein or 12 μmoles CHO / μmole protein indicating that canine pancreatic calreticulin may be glycosylated but at a very low level as compared to its homologue calsequestrin. By the method of Dubois *et al.* (1956) it appeared that calreticulin had 3.5 times less CHO content that canine pancreatic calreticulin. With such a small CHO content the changes upon glycosidase treatment could not be observed. It was shown that rat liver calreticulin is composed of a complex hybrid type of oligosaccharide with a terminal galactose residue (Van *et al.*, 1989; Peter *et al.*, 1992), a type of glycosylation which is very unusual for resident ER proteins. If such a complex glycosylation pattern was present in pancreatic calreticulin, it may explain why the glycosidases failed to cleave away the CHO residues and thus failed to produce a shift detectable by SDS-PAGE. Since the rat liver calreticulin contained a terminal galactose the protein must, therefore, pass through the trans-Golgi before being transported back to the ER. Söling's group had shown that this terminal galactosylation of calreticulin was abolished when the vesicular transport from the intermediate- to trans-Golgi was blocked (Peter *et al.*, 1992). It was postulated that retention of calreticulin mediated by KDEL-receptor must extend into the Golgi, including the trans-Golgi (Peter *et al.*, 1992). Further studies are required to establish firmly the glycosylation patterns of calreticulin and the precise trafficking of this protein between different cellular compartments. Glycosylation in calreticulin is species specific and possibly a tissue specific event and may affect the localization of the protein, but may not be involved directly in its function.

Phosphorylation of Calreticulin

As mentioned earlier, calreticulin has several protein kinase C recognition sequences (clustered at the N-domain of the protein: residues 17-19, 36-38, 61-63, 68-70, 79-81 and 124-126), casein kinase II (residues 51-54, 172-175, 178-181, 196-200, 204-208, 307-311, and 316-319) and tyrosine kinase (residues 261-268). We failed to show any phosphorylation of either to native or recombinant calreticulin by protein kinase C, cAMP-dependent protein kinase, casein kinase II or tyrosine kinase (C. Shemanko, S. Baksh, R. E. Milner and M. Michalak, unpublished work). Calreticulin also appeared to have a sequence with marked similarity to the active site of protein kinase C (215-224: KPEDWDKPEH). Using histones as substrate for protein kinase C phosphorylation, calreticulin did not have a detectable kinase activity. This was not entirely surprising since calreticulin does not contain an ATP binding site. Calreticulin, as a phosphoprotein, interacted with the 3' Cis-acting element of the rubella virus RNA molecule (Singh *et al.*, 1994). Viral infection of Vero 76 cells promoted the phosphorylation of the simian calreticulin. Phosphorylation of calreticulin, similar to glycosylation, is species-specific and appears to be enhanced during viral infection.

Staphylococcus aureus V8 protease peptide map of calreticulin

Peptide mapping using proteolytic enzymes can yield useful information concerning accessibility of residues under different assay conditions. SDS-PAGE analysis of the proteolytic digestion of canine pancreatic calreticulin using Staphylococcus aureus V8 protease is documented in Fig. 3-14A. V8 protease cleaves on the carboxyl-side of aspartic (D) and glutamic (E) residues (Cleveland *et al.*, 1977). Digestion in the absence of Ca^{2+} or Zn^{2+} (Fig. 3-14A, lanes b and e) produced a mixture of peptide fragments, with a 21 kDa fragment being the most predominant. In the presence of 1 mM Ca^{2+} (Fig. 3-14A, lane c), we did not observe a change in the peptide map indicating that the binding of Ca^{2+} to calreticulin does not shift accessibility of D or E residues. V8 digestion did appear to proceed at the same rate with or without Ca^{2+} bound to the protein. Zn^{2+}, however, confered a small degree of protection of D and E residues since we observed a small amount of undigested calreticulin at the top of lane f. We still observed the predominant 21 kDa band in the presence of Zn^{2+}. Therefore, the overall positions of D or E residues might be different in the presence of Ca^{2+} or Zn^{2+}, with Zn^{2+} creating a greater movement of D or E residues and offering a degree of protection against digestion with Staphylococcus aureus V8 protease. Thus, Zn^{2+} caused a relatively greater change in calreticulin's secondary structure than Ca^{2+}. This correlated with previous fluorescence data by Khanna *et al.* (1986), who demonstrated that calreticulin showed a greater change in its intrinsic fluorescence pattern in the presence of Zn^{2+} than in the presence of Ca^{2+}.

RNA binding to calreticulin

The preparations of canine pancreatic calreticulin have been found to contain a significant amount of nucleotide signal associated with it. Fig. 3-14B documented the isolation of the associated nucleotide component of canine pancreatic calreticulin. The nucleotide associated with calreticulin migrated on a DNA agarose gel at the level of the 60 - 100 bp marker (Fig. 3-14B). This nucleotide species was associated with calreticulin at the DEAE (Fig. 3-14B, lane f), and at the hydroxylapatite stage of calreticulin's protein purification (Fig. 3-14B, lane c). Separation of the nucleotide from calreticulin was only attainable on a Zn^{2+}-chelating Sepharose column, whereby the nucleotide species appeared in the flowthrough fractions (Fig. 3-14B, lanes j and k), but not in the eluted fractions (Fig. 3-14B, lane l). The eluted fraction was known to contain calreticulin (Chapter Five: Identification of the Zn^{2+} binding sites within calreticulin). In order to further establish the identity of this molecule, RNase A and DNase I digestion were carried out. The nucleotide sample digested only with RNase A (Fig. 3-14C, lane b) and not with heat treated DNase I (Fig. 3-14C, lane d). Identification of this RNA species awaits chemical nucleotide sequencing of the molecule. Calreticulin is known to associate with two distinct RNA species - the rubella virus RNA (Singh *et al.*, 1994) and the Ro/SS-A hY RNA species (Deutscher *et al.*, 1988). Preliminary Northern Blot analysis failed to show hybridization to the hY5 RNA species of the Ro/SS-A complex. Once the identity of this RNA species is determined, further experiments will have to carried out to

Figure 3-14: Staphylococcus aureus V8 protease peptide map of calreticulin and characterization of RNA species associated with calreticulin

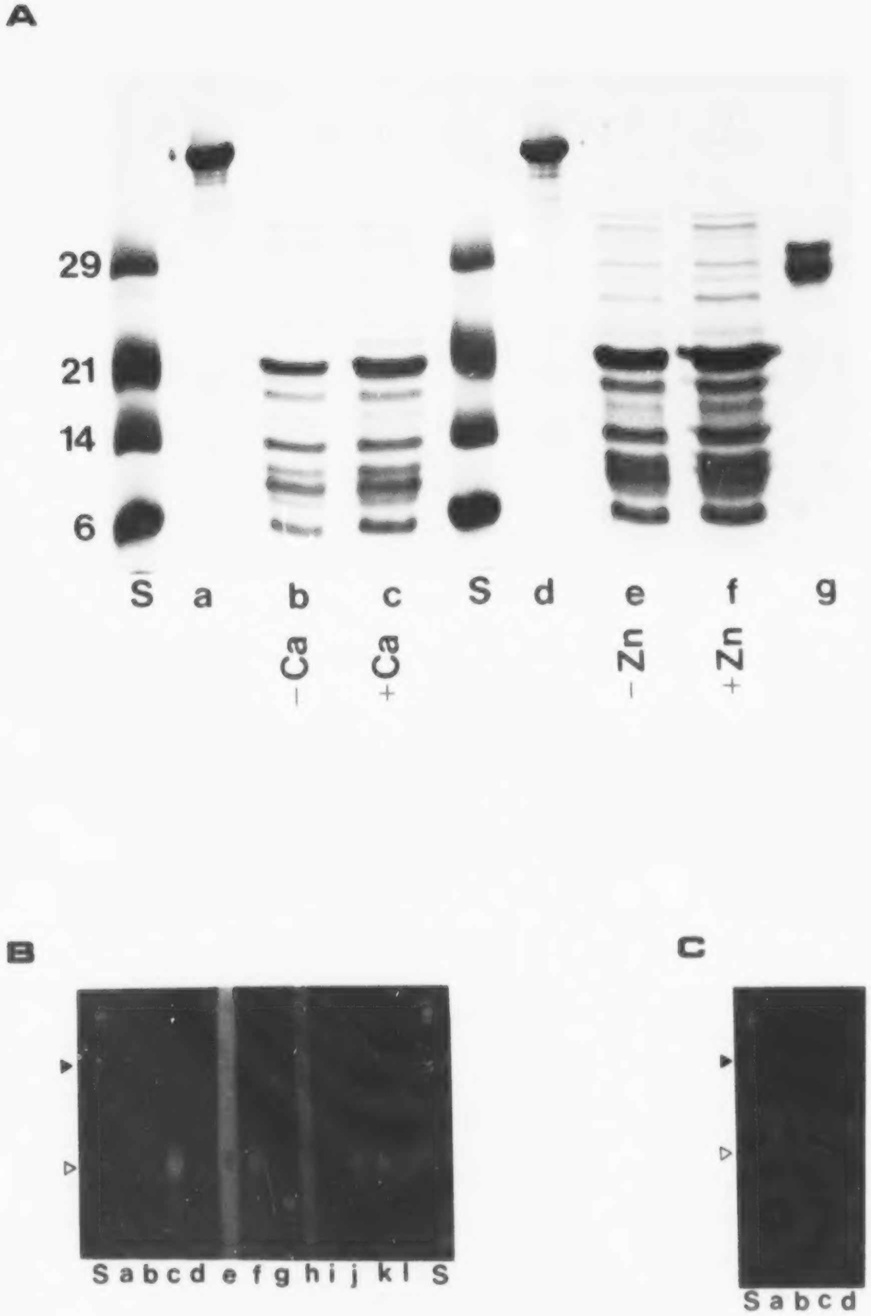
(A) Staphylococcus aureus V8 protease peptide map of calreticulin (10 µg) in the presence of 1 mM Ca^{2+} or 100 µM Zn^{2+}. Samples were digested as described in "Chapter Two: Experimental Procedures" and then separated by SDS-PAGE (15% gel) followed by Coomassie Blue staining. Lanes a and d, dog pancreatic calreticulin; lanes b and e, dog pancreatic calreticulin digested with V8 protease (1 : 5 enzyme to protein ratio); lane c, dog pancreatic calreticulin digested with V8 protease (1 : 5 enzyme to protein ratio) **in the presence of 1 mM Ca^{2+}**; lane f, dog pancreatic calreticulin digested with V8 protease (1 : 5 enzyme to protein ratio) **in the presence of 100 µM Zn^{2+}**; lane g, V8 protease (5 µg). S - Mandel low molecular weight protein standards as indicated.

(B) *Identification of the RNA species associated with calreticulin during the different stages of the calreticulin purification*

The hydroxylapatite peak (fractions 15 - 17 of Fig. 3-2) was loaded onto a Zn^{2+} chelating column and the bound proteins were eluted with an imidazole gradient (0 --> 50 mM imidazole). The flowthrough and eluted fractions were separated on a 12% DNA agarose gel and ethidium bromide stained to visualize the bands. Lane S, λ–ϕ DNA standards; lane a and d, recombinant calreticulin standard; lane b, dog pancreatic calreticulin standard; lane c, hydroxylapatite calreticulin peak; lane e and f, DEAE calreticulin peak (fractions 31-47, Fig. 3-1); lane g, flowthrough from hydroxylapatite column (not containing calreticulin); lane h and i, eluted fractions 15 and 17 from hydroxylapatite column (containing calreticulin, Fig. 3-2); lane j and k, fractions 5 and 6 of flowthrough from Zn^{2+} chelating column (containing RNA); lane l, eluted fractions 9 and 13 from the Zn^{2+} chelating column (containing calreticulin).

(C) *Digestion of the flowthrough fractions of the Zn^{2+} chelating column (containing RNA) with RNase A and DNase I*

Lane S, λ–ϕ DNA standards; lane a, flowthrough fractions from the Zn^{2+} chelating column; lane b, flowthrough fractions from the Zn^{2+} chelating column **digested with RNase A**; lane c, flowthrough fractions from the Zn^{2+} chelating column **digested with DNAase I**; lane d and e, flowthrough fractions from the Zn^{2+} chelating column **digested with heat treated DNase I**. The position of the 300 bp DNA marker is indicated with the closed triangle and the position of the 60 bp marker is indicated with the open triangle.


A
29
21
14
6
S a b c S d e f g
−Ca
+Ca
−Zn
+Zn
B
S a b c d e f g h i j k l S
C
S a b c d

determine its interaction with calreticulin and how it may modulate calreticulin's function within the cell.

DISCUSSION

Analysis of the post-translational processing events of glycosylation and phosphorylation revealed that the canine pancreatic form of calreticulin does not undergo any detectable post-translational processing. Canine pancreatic calreticulin isolated via the ammonium sulfate precipitation method and was found to be glycosylated at a low level as compared to canine cardiac calsequestrin. It was not found to be phosphorylated by protein kinase C, protein kinase A, or casein kinase II.

Biochemical characterization using Stains-All, revealed that both C- and P-domains were responsible for the blue staining of mature calreticulin with Stains-All (Fliegel *et al.*, 1989b; Krause *et al.*, 1990; Milner *et al.*, 1991) and that this may be the localization of the two Ca^{2+} binding sites within the protein. Several other high and low affinity Ca^{2+} binding proteins have been identified by their blue staining with Stains-All, including calmodulin, troponin C, S-100, 160-kDa sarcalumenin, calsequestrin and calreticulin (Jones and Cala, 1981; Campbell *et al.*, 1983; Fliegel *et al.*, 1989b; Krause *et al.*, 1990; Treves *et al.*, 1990; Milner *et al.*, 1991). The Ca^{2+} binding sites in calmodulin and troponin C are of the EF hand type (Heizmann and Hunziker, 1991), whereas in calsequestrin and calreticulin they are not (MacLennan *et al.*, 1983; Ohnishi and Reithmeier, 1987; Fliegel *et al.*, 1989a, Smith and Koch, 1989). Both types of Ca^{2+} binding protein stain blue with Stains-All indicating that the blue color is unrelated to the specific type of Ca^{2+} binding site (Campbell *et al.*, 1983).

Calreticulin's structure has not been determined yet. Based on sedimentation equilibrium analysis, it was found to exit as a monomer, and disulfide analysis revealed the possible presence of an intramolecular 25-amino acid loop within the highly structured NH_2-terminus. Preliminary scanning tunneling electron microscopy (STEM) data indicate that calreticulin has the potential for the formation of oligomers. The influence of Ca^{2+} on the possible oligomerization process still remains to be tested. Once the structure of this novel Ca^{2+} binding protein of the endoplasmic reticulum of non-muscle cells has been solved, a better understanding of the varied functions that it may carry out will be understood.

CHAPTER FOUR

Identification of the calcium binding sites within calreticulin

A version of this chapter has been published - Baksh, S. and Michalak, M. 1991 *J. Biol. Chem.* 266: 21458 - 21465.

INTRODUCTION

Calreticulin is a Ca^{2+} binding protein which was first identified almost 20 years ago by Ostwald and MacLennan (1974) as the high affinity Ca^{2+} binding protein of skeletal muscle SR membranes. This protein is, however, only a minor component of the SR in skeletal (Michalak *et al.*, 1980) and cardiac muscle (Fliegel *et al.*, 1989b) and in these tissues the major Ca^{2+} binding/storage protein is considered to be calsequestrin (for review see MacLennan *et al.*, 1983). In contrast, calreticulin has been identified as a major Ca^{2+} binding (storage) protein of smooth muscle SR and non-muscle ER (Milner *et al.*, 1991). Both calreticulin and calsequestrin are high capacity Ca^{2+} binding proteins; calsequestrin binds 30-50 moles of Ca^{2+} per mole of protein with a low affinity of approximately 1 mM (MacLennan *et al.*, 1983). MacLennan's group have shown that calreticulin, in addition to having a single high affinity site may also possess a number of low affinity Ca^{2+} binding sites (MacLennan *et al.*, 1972; Ostwald and MacLennan, 1974). On the basis of this observation, and the tissue distribution of the two proteins, calreticulin may be a non-muscle functional analogue of calsequestrin (Milner *et al.*, 1991).

Calsequestrin (Fliegel *et al.*, 1987; Scott *et al.*, 1988) and calreticulin (Fliegel *et al.*, 1989a; Smith and Koch, 1989; McCauliffe *et al.*, 1990a; Murthy *et al.*, 1990) have both been cloned and their amino acid sequences deduced from the cDNAs encoding them. Importantly, the only similarity in the amino acid sequences of these two proteins occurs in the clusters of acidic residues found at their respective carboxyl-termini and even this similarity is limited (Fliegel *et al.*, 1989a). Calreticulin has been proposed to be divided structurally into distinct domains (Fliegel *et al.*, 1989b; Smith and Koch, 1989) - the hydrophobic N-domain, which leads into the proline-rich P-domain followed by the acidic C-domain. The C-domain of calreticulin has 37/57 residues which are aspartic or glutamic acid and also terminates with the KDEL ER retention signal. The functional implications of these distinct domains within the sequence of calreticulin are not clear at present. However, in analogy with observations on the Ca^{2+} binding behavior of calsequestrin (Ohnishi and Reithmeier, 1987) we proposed that the C-domain of calreticulin might contain the low affinity and high capacity Ca^{2+} binding site. Although a high affinity Ca^{2+} binding site has been identified in calreticulin (Ostwald and MacLennan, 1974) the protein amino acid sequence contains no EF-hand consensus sequences (Fliegel et al., 1989a; Smith and Koch, 1989), and, therefore, the location of this Ca^{2+} binding site in the protein has yet to be determined.

In Chapter three, I have described the expression in E. coli. of recombinant calreticulin and calreticulin domains using a plasmid vector which allowed for a high level of expression of calreticulin fusion proteins with the enzyme GST (Smith and Johnson, 1988). In the present study, we have used the GST fusion proteins in order to identify the domains involved in Ca^{2+} binding to this protein. This study should provide important information concerning the structural requirements associated with high affinity Ca^{2+} binding sites in proteins lacking the EF-hand consensus motif.

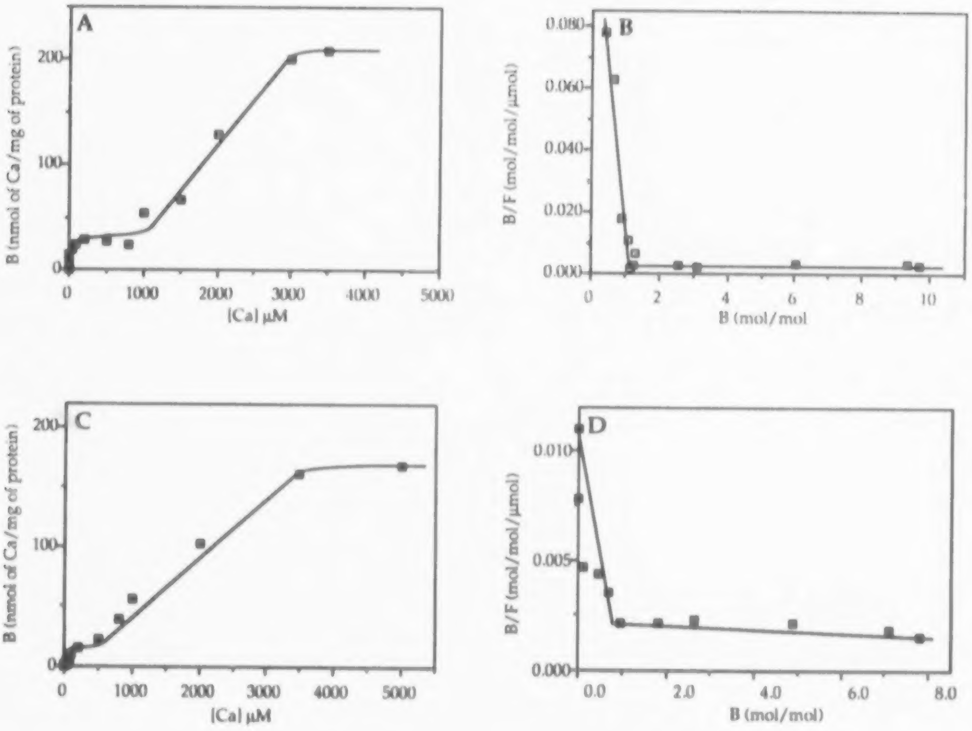
RESULTS

Ca^{2+} Binding to Native and Recombinant Calreticulin - Ca^{2+} binding to purified native canine pancreatic (A,B) and recombinant calreticulin (C,D) was determined by an equilibrium dialysis assay (Fig. 4-1). Using at least a 10 000-fold range of Ca^{2+} concentration we found that native calreticulin bound Ca^{2+} with high affinity (11.4 μM) and low capacity (1 mole/mole of protein) as well as with low affinity (2 mM) and high capacity (17 moles/mole of protein) (Fig. 4-1A & B). This is in agreement with earlier observations (Ostwald and MacLennan, 1974). Importantly, both Ca^{2+} binding sites were present in the recombinant protein indicating that proper folding of calreticulin was achieved (Fig. 4-1C & D). Ca^{2+} binding to the low affinity site (approximately 21 moles of Ca^{2+}/ mole of protein) exhibited a binding constant of approximately 2 mM. The high affinity Ca^{2+} binding site of the recombinant calreticulin exhibited a maximal binding capacity of approximately 1 mole of Ca^{2+}/mole of protein with a binding constant of approximately 11 μM.

Ca^{2+} binding to the low affinity high capacity site in calreticulin was reduced by approximately 60% when measured in the presence of 3 mM Mg^{2+}. The high affinity low capacity site, however, was not effected by the inclusion of 3 mM $MgCl_2$. This observation probably explains why Van *et al.* (1989) and Waisman *et al.* (1987) did not detect a low affinity, high capacity Ca^{2+} binding site in calreticulin, since these authors included high concentrations of Mg^{2+} in the buffers used for Ca^{2+} binding studies.

Figure 4-1: Ca^{2+} Binding to native and recombinant calreticulin

Ca^{2+} binding to native (A,B) and recombinant calreticulin (C,D) (300-600 µg) was carried out by equilibrium dialysis as described under "Chapter Two: Experimental Procedures." SDS-PAGE of the proteins used for Ca^{2+} binding is shown in Figure 3-3A lanes 4 and 5, respectively. Scatchard analysis of the Ca^{2+} binding data is presented for the native calreticulin (B) and the recombinant calreticulin (D). B, bound; B/F, bound/free. The data shown in this figure represent the mean of values measured in triplicate using two different protein preparations.

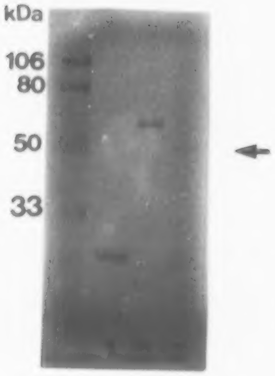

A
B (nmol of Ca/mg of protein)
0
100
200
0
1000
2000
3000
4000
5000
[Ca] µM
B
B/F (mol/mol/µmol)
0.000
0.020
0.040
0.060
0.080
0
2
4
6
8
10
B (mol/mol)
C
B (nmol of Ca/mg of protein)
0
100
200
0
1000
2000
3000
4000
5000
[Ca] µM
D
B/F (mol/mol/µmol)
0.000
0.005
0.010
0.0
2.0
4.0
6.0
8.0
B (mol/mol)

Ca^{2+} Binding to the Fusion Proteins - The N-, P-, and C-domain of calreticulin were tested for Ca^{2+} binding in order to determine the localization of the high capacity, low affinity sites and the high affinity, low capacity site. Figure 4-2C showed that the N-domain fusion protein (lane 1) and purified GST (lane 1) did not bind any Ca^{2+} under the $^{45}Ca^{2+}$ overlay conditions, whereas purified native calreticulin bound Ca^{2+} (lane 2) under these conditions. Fig. 4-3A showed Ca^{2+} binding to the recombinant C-domain and P-domain. The recombinant C-domain bound up to 18 moles of Ca^{2+} per mole of protein (Fig. 4-3A) and did not bind significant amounts of Ca^{2+} at the Ca^{2+} concentrations below 300 µM. This result indicated that the high capacity Ca^{2+} binding site of calreticulin is located in the C-domain of the protein. In contrast, the P-domain bound maximum levels of only 0.6 - 1 mole of Ca^{2+} per mole of protein (Fig. 4-3A). This binding was saturated between 300 to 500 µM Ca^{2+} concentration. The N+P-domain showed the same binding properties as the P-domain (Table 4-1).

In order to further analyze Ca^{2+} binding to calreticulin the P- and C-domains were expressed as one entity (P+C-domain). Based on the results using the C-domain and P-domain fusion proteins, this domain should contain both the high affinity and the low affinity Ca^{2+} binding sites. Figure 4-3B showed Ca^{2+} binding to the P+C-domain. As expected this domain exhibited both Ca^{2+} binding characteristics of the native and recombinant proteins, binding Ca^{2+} with high affinity (6 µM) and low capacity (1.3 mole of Ca^{2+}/mole of protein), and with low affinity (2 mM) and high capacity (18 moles of Ca^{2+}/mole of protein) (Fig. 4-3B). The constants associated with Ca^{2+} binding to native and to calreticulin

Figure 4-2: Ca^{2+} Binding to the N-domain of calreticulin

The GST-N-domain was separated by SDS-PAGE (10 % gel) (A), transferred electrophoretically to nitrocellulose membranes and then immunoblotted with the rabbit anti-GST antibody (B) or incubated with $^{45}Ca^{2+}$ (C) as described under "Chapter Two: Experimental Procedures." Lane 1, GST; lane 2, dog pancreatic calreticulin; lane 3, GST-N-domain. S, molecular weight standards as indicated. The GST-N-domain is indicated by the arrow. The appearance of the Mr standards in C is due to the light emission from the dye bound to the standards proteins.


kDa
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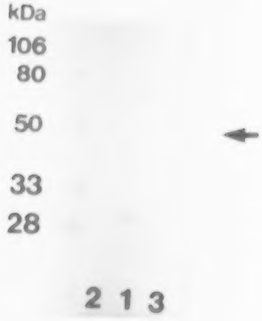
80
50
33


kDa
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50
33
28
2 1 3

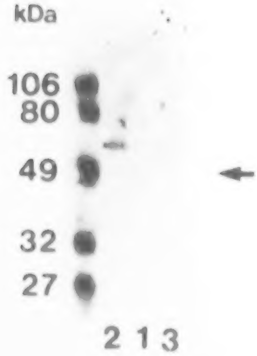
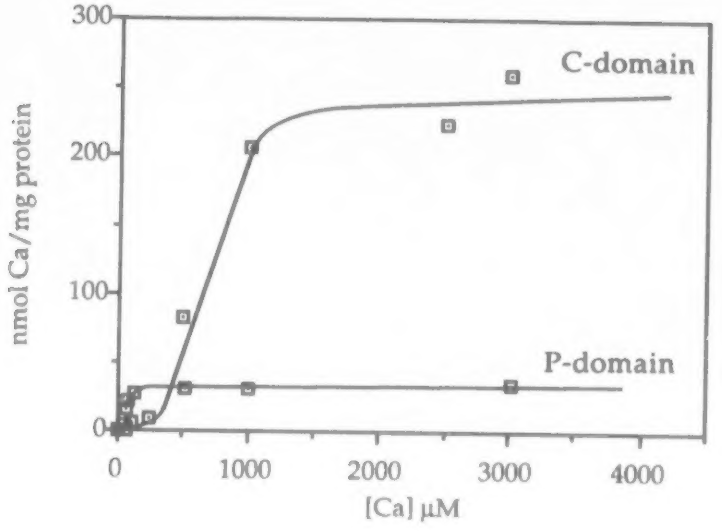

kDa
106
80
49
32
27
2 1 3

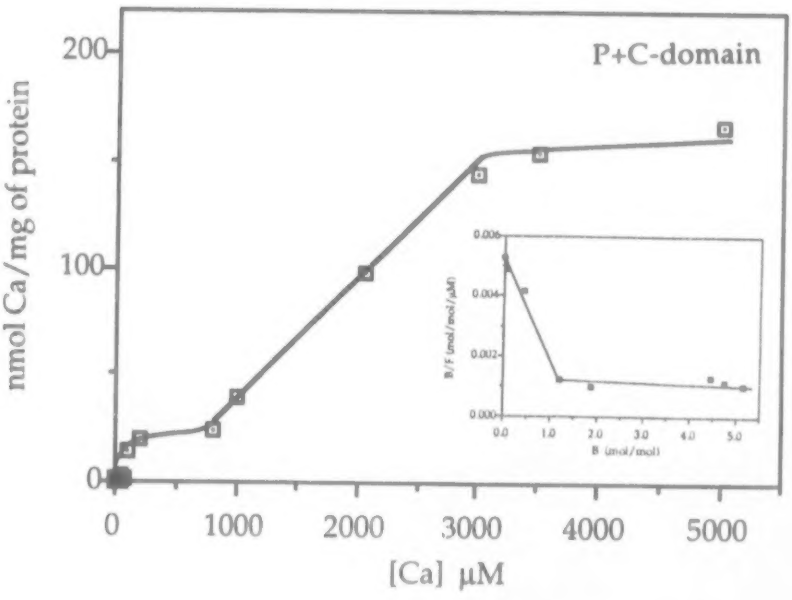
Figure 4-3: Ca^{2+} Binding to the P- and C-domain Fusion Proteins

Ca^{2+} binding to GST-fusion proteins was carried out by equilibrium dialysis as described under "Chapter Two: Experimental Procedures." (A) Ca^{2+} binding to the C-domain (600 µg) and to the P-domain (300 µg); (B) Ca^{2+} binding to the GST-P+C-domain (300-600 µg). Inset - Scatchard analysis of Ca^{2+} binding data. B, bound; B/F, bound/free. SDS-PAGE of the C-, P-domains, and GST-P+C-domain used for Ca^{2+} binding are shown in Figure 3-5B lanes f, h, and l respectively. The data shown in this figure represent the mean of values measured in triplicate using two different protein preparations.

A


C-domain
P-domain
nmol Ca/mg protein
300
200
100
0
0
1000
2000
3000
4000
[Ca] µM

B


P+C-domain
nmol Ca/mg of protein
200
100
0
0
1000
2000
3000
4000
5000
[Ca] µM
B/F (mol/mol/µM)
0.006
0.004
0.002
0.000
0.0
1.0
2.0
3.0
4.0
5.0
B (mol/mol)

domains are summarized in Table 4-1.

Conformational changes upon Ca^{2+} binding - Circular dichroism analysis was carried out on calreticulin in the absence and presence of Ca^{2+} in order to determine possible secondary structural changes upon Ca^{2+} binding. Upon the binding of Ca^{2+}, very subtle changes in the secondary structure was observed (Fig. 4-4, far UV spectrum). This was similar to the observation by Khanna *et al.* (1986). A decrease in $[ø]_{222}$ from -6 400 deg.cm^{2}dmol^{-1} to -7800 deg.cm^{2}dmol^{-1} occurred in the far UV spectrum. This wavelength is characteristic in representing the α-helical secondary structural element of a protein's structure. However, an increase in the ellipticity was observed for the near UV spectrum. This observation was likely a summation of the contributions of the aromatics residues within calreticulin (mainly tryptophan, tyrosine, and phenylalanine). The observed increase in ellipticity was indicative of movement of aromatics into the solvent and a concomitant loss of hydrophobicity. These changes upon binding of Ca^{2+} were again very small and appear to be very local. Calsequestrin, however, undergoes a considerable change in secondary structural upon Ca^{2+} binding (Ostwald and MacLennan, 1974). It thus appeared that even though both calreticulin and calsequestrin have similar carboxyl-terminal regions, the structure of their high capacity, low affinity Ca^{2+} binding sites are different resulting in different secondary structural changes upon binding of Ca^{2+}.

Table 4-1:

Ca^{2+} binding to calreticulin and calreticulin domains

Protein	High affinity site		High capacity site	
	K_d µM	B_{max} mol Ca^{2+}/mol of protein	K_d mM	B_{max} mol Ca^{2+}/mol of protein
Native calreticulin	11.4	1.3	8.7	20.4
Recombinant calreticulin	11.0	1.1	7.0	21.0
P-domain	10.9	0.6	---	---
C-domain	---	---	9.0	18
N+P-domain	10.6	1.3	---	---
P+C-domain	6.1	0.7	8.8	17.5

Note: For native calreticulin, fraction #17 from the hydroxylapatite column was used (see Fig. 3-2); recombinant calreticulin was taken from fraction #24 of the Mono Q FPLC column (see Fig. 3-4A). See Fig. 3-5B for SDS-PAGE analysis of samples of calreticulin domains: P-domain, lane h; C-domain, lane f; N+P-domain, lane n; P+C-domain, lane l. All calreticulin domain samples were purified on a Glutathione S-transferase affinity column. "---"denotes no Ca^{2+} binding to the protein.

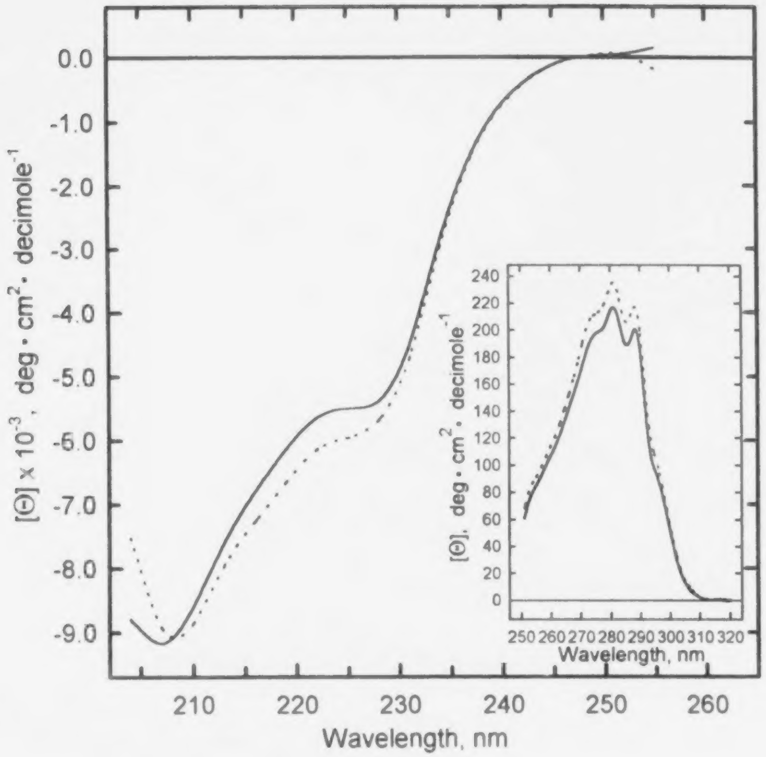


Figure 4-4: Ca^{2+}-Dependent Conformational Changes in Calreticulin

CD measurements were carried out as described under "Chapter Two: Experimental Procedures". Far UV CD spectra of calreticulin is shown in the absence (—) and presence of 2 mM Ca^{2+} (·····). Inset; near UV CD spectra of calreticulin in the absence (—) and presence of 2 mM Ca^{2+} (····).

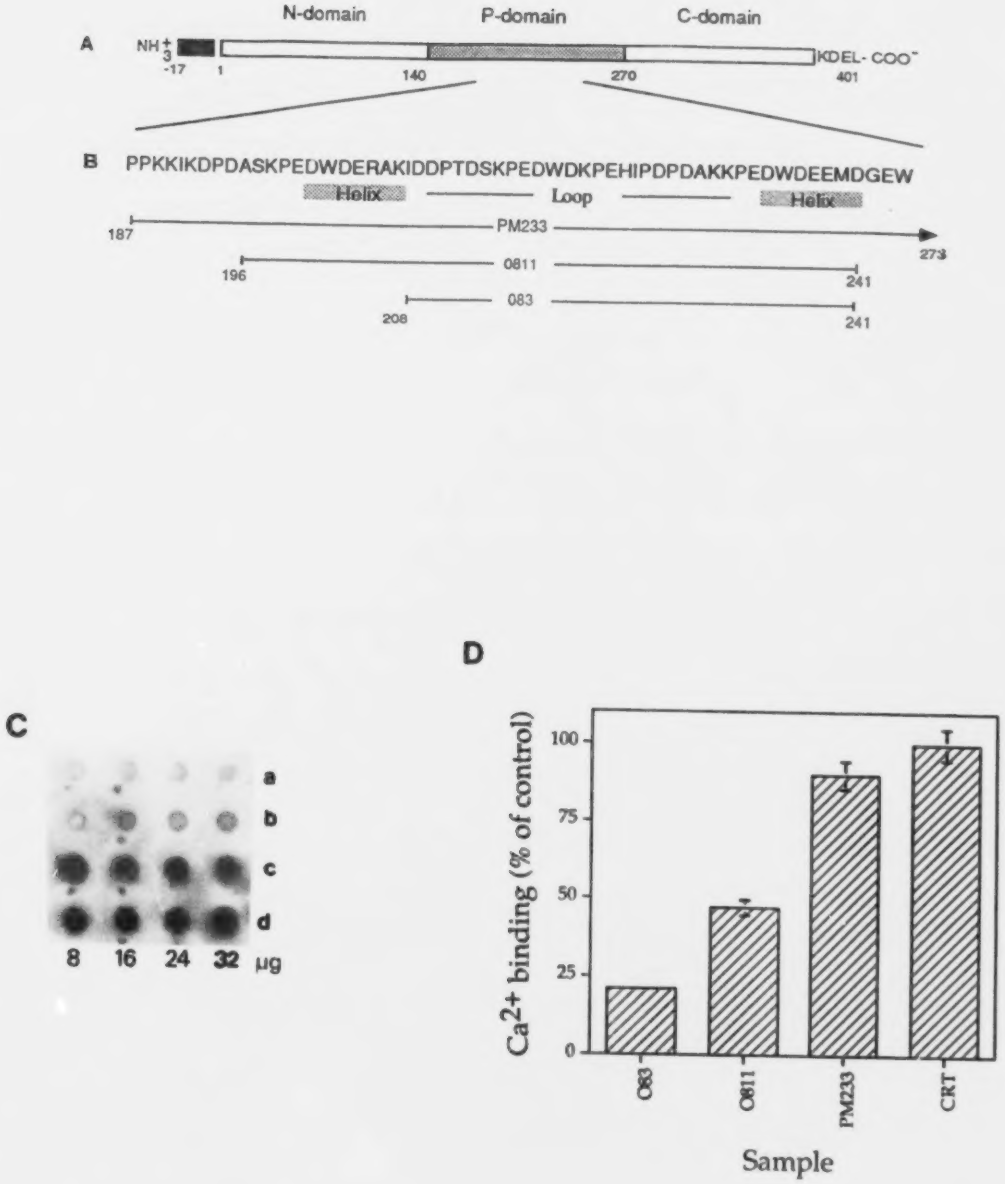
Analysis of the high affinity Ca^{2+} binding site

Use of synthetic peptides - The P-domain of calreticulin was shown to contain the high affinity Ca^{2+} binding site (Fig. 4-3A). The next stage of this study was to determine what were the residues involved in binding of Ca^{2+} to this domain of calreticulin. As mentioned earlier, calreticulin does not contain the EF-hand consensus amino acid sequence found in other high affinity Ca^{2+} binding proteins such as calmodulin and troponin C (Kretsinger, 1988). The EF-hand Ca^{2+} binding site forms a helix-loop-helix motif (Kretsinger, 1988). Computer algorithm analysis of the amino acid sequence of the P-domain (Fig. 3-9A) predicted that a helix-loop-helix structural motif was found within the P-domain of calreticulin (Fig. 4-5B). The amino acid sequence spanning the helix-loop-helix structural motif contained the three repeat sequence A (Fig. 1-2B) found in all calreticulins and in eukaryotic calnexin. We hypothesized that this helix-loop-helix motif may be responsible for high affinity Ca^{2+} binding to calreticulin. Two approaches were undertaken to identify the high affinity Ca^{2+} binding site within the P-domain: Ca^{2+} binding was analyzed using synthetic peptides and truncated P-domain fragments. Figure 4-5B described the amino acid sequence of the two synthetic peptides and the truncated P-domain fragment (PM233) used in these studies and their corresponding location within the P-domain. Increased amounts of the 083 peptide (Fig. 4-5C, a), the 0811 peptide (Fig. 4-5C, b), the truncated P-domain fragment (PM233) (Fig. 4-5C, c), and native calreticulin (Fig. 4-5C, d) were spotted onto nitrocellulose membrane and tested for $^{45}Ca^{2+}$ binding via the $^{45}Ca^{2+}$ overlay technique. All the

Figure 4-5: Amino acid sequence of the synthetic peptides used for the identification of the high affinity Ca^{2+} binding site within calreticulin's P-domain

(A, B) Peptides 083 and 0811 were synthesized by the PENCE group at the University of Alberta and was a generous gift of Dr. Hodges. PM233 was obtained by factor Xa cleavage of the GST-P-domain fusion protein (Fig. 3-8A) as described in "Chapter Two: Experimental Procedures". (A) Schematic of domain structure of calreticulin; (B) Partial Sequence of the P-domain region. Numbers indicate positions within the amino acid sequence of rabbit calreticulin (Fliegel *et al.*, 1989b).

(C,D) Eight, 16, 24, 32 µg of the synthetic peptides, the truncated P-domain fragment (PM233), and calreticulin were blotted onto nitrocellulose membranes followed by incubation with $^{45}Ca^{2+}$ (C). The 24 µg radioactive dots of (C) were scanned using a Chromoscanner 5 densitometric scanner and then quantitated (S. D. ± 2.3; n = 2). A bar graph showing these results is given in (D). In C: a, peptide 083; b, peptide 0811; c, PM233; d, dog pancreatic calreticulin. Micrograms of protein used is indicated. Ca^{2+} binding to calreticulin was used as 100% value for Ca^{2+} binding. This data is the average of two experiments.


A
N-domain
P-domain
C-domain
NH$_3^+$
-17
1
140
270
401
KDEL- COO$^-$
B
PPKKIKDPDASKPEDWDERAKIDDPTDSKPEDWDKPEHIPDPDAKKPEDWDEEMDGEW
Helix
Loop
Helix
187
PM233
273
196
0811
241
208
083
241
C
a
b
c
d
8
16
24
32
µg
D
Ca^{2+} binding (% of control)
0
25
50
75
100
O83
O811
PM233
CRT
Sample

samples bound $^{45}Ca^{2+}$ via the $^{45}Ca^{2+}$ overlay technique. The 083 peptide had the weakest binding suggesting that it did not contain the entire Ca^{2+} binding site. Although all three repeat sequence A segments are present in the synthetic peptide 0811, it bound more Ca^{2+} than the synthetic peptide 083, but significantly less than the truncated P-domain fragment PM233, indicating that flanking residues on either sides of the first and third repeat sequence A may be important for high affinity Ca^{2+} binding to this domain.

Tryptophan fluorescence on both the 083 peptide and PM233 showed no discernible changes in the tryptophan environment of these samples (significant amounts of 0811 was not available to perform the tryptophan fluorescence experiments). Circular dichroism analysis on PM233 in the presence of Ca^{2+}, however, revealed small changes in the aromatic ellipticity (Fig. 4-6) and a small quenching of the ellipticity that was indicative of movement of aromatic residues away from the solvent and assuming a more hydrophobic shape. This was opposite to what was observed for calreticulin upon Ca^{2+} binding (compare inset between Fig. 4-4 and Fig. 4-6). The main contribution to the aromatic spectrum in Fig. 4-6 was the presence of eight tryptophan residues within the truncated P-domain fragment PM233 since this region only has one phenylalanine residue and no tyrosine residues. It appears that within the truncated P-domain fragment PM233, two distinct populations of tryptophan residues were present - one population that did not undergo a change in ellipticity when Ca^{2+} is added (positive bands at 297 nm and 287 nm), and a second population that showed a decrease in ellipticity and movement away from the solvent (positive bands at 282 nm and 270 nm). The

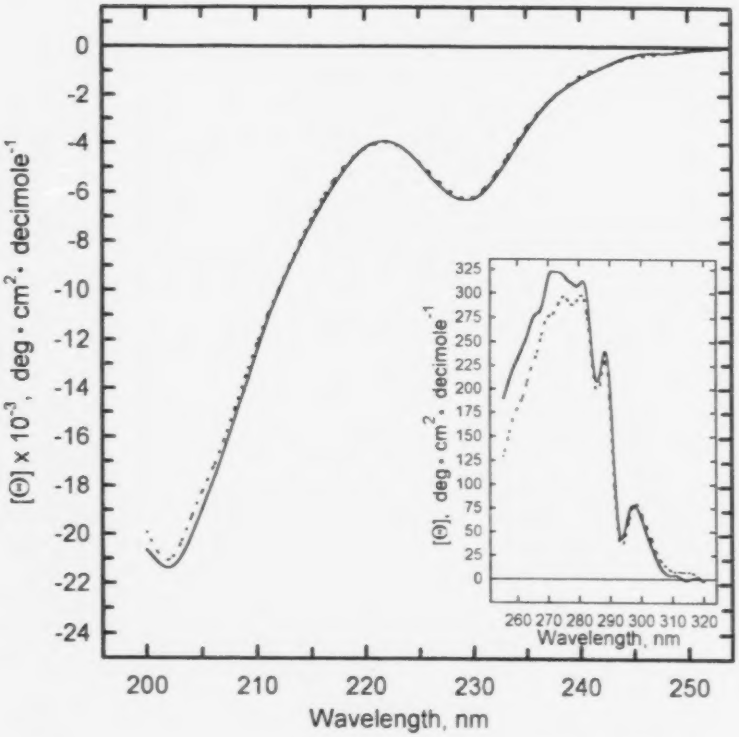


Figure 4-6: Ca^{2+} dependent conformational changes in the truncated P-domain fragment PM233

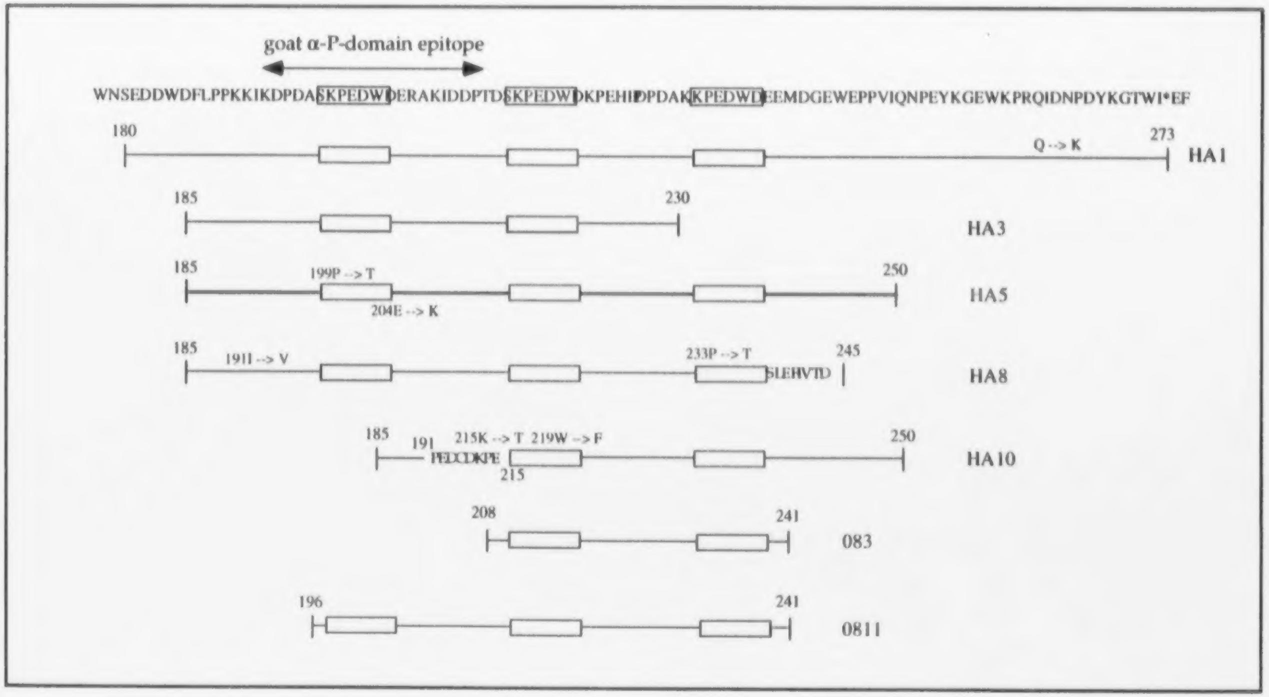
CD measurements of the truncated P-domain fragment PM233 were carried out as described under "Experimental Procedures". Far UV CD spectra of the truncated P-domain (PM233) in the absence (—) and presence of 1 mM Ca^{2+} (····). Inset; near UV CD spectra of the truncated P-domain fragment PM233 in the absence (—) and presence of 1 mM Ca^{2+} (····).

insignificant change in the far UV spectrum indicated that the peptide backbone remained unchanged, whilst changes in the near UV certainly indicated surface movements of specific aromatic residues, mainly the tryptophan residues. This observation implicated the importance of tryptophan residues in high affinity Ca^{2+} binding.

Analysis of the truncated GST-P-domain fusion proteins - Studies involving synthetic peptides revealed that fragments longer than 45 amino acids (i. e., peptide 0811) (Fig. 4-5B), but shorter than 82 amino acids (the truncated P-domain fragment PM233) (Fig. 3-8B) may be involved in high affinity Ca^{2+} binding to the P-domain of calreticulin. GST-fusion proteins were constructed to further identify the location of the high affinity Ca^{2+} binding site within the P-domain of calreticulin. The hypothesis was that all three repeat sequence A segments may be responsible for providing the helix-loop-helix structure shown in Fig. 4-5B and that the motif may be responsible for high affinity Ca^{2+} binding to the P-domain of calreticulin. Figure 4-7 is a diagrammatic representation of the truncated GST-P-domain fusion proteins used in this study. The boxed areas are location of the KPEDWD repeat sequence found within repeat sequence A; letters above the lines indicate mutational changes within these areas. The GST-fusion proteins were purified by one step affinity chromatography on Glutathione-Sepharose affinity chromatography and then analyzed by SDS-PAGE (Fig. 4-8A), followed by immunostaining with the affinity purified goat anti-P-domain antibody (Fig. 4-8B) and $^{45}Ca^{2+}$ overlay analysis (Fig. 4-8C).

Figure 4-7: Amino acid sequence of truncated GST-P-domain fusion proteins used to study high affinity Ca^{2+} binding to calreticulin

The partial amino acid sequence of the P-domain of calreticulin in shown (the full length P-domain is from 170 - 273). Truncated P-domain proteins were expressed as GST-fusion proteins and were designated GST-HA1, GST-HA3, GST-HA5, GST-HA8, and GST-HA10. Boxed areas represent the position of the KPEDWD repeat sequence present within repeat sequence A (Fig. 1-2B). Isolated letters indicated amino acid changes from the wild type rabbit sequence (Fig. 1-2A). 083 and 0811 are synthetic peptides described in Fig. 4-5A. The epitope specificity of the goat-anti-P-domain specific antibody is shown (this was determined from results in Fig. 4-8B). The GST-HA10 fusion protein contained only the last two repeat sequence A repeats and 13 flanking amino acids after the second repeat. This fusion construct contained amino acids 185 to 191, followed by the sequence PEDCDKPE and then continued from amino acid 215 to 250. GST-HA8 contained all three repeat sequence A repeats and the sequence SLEFIVTD immediately after the third repeat.

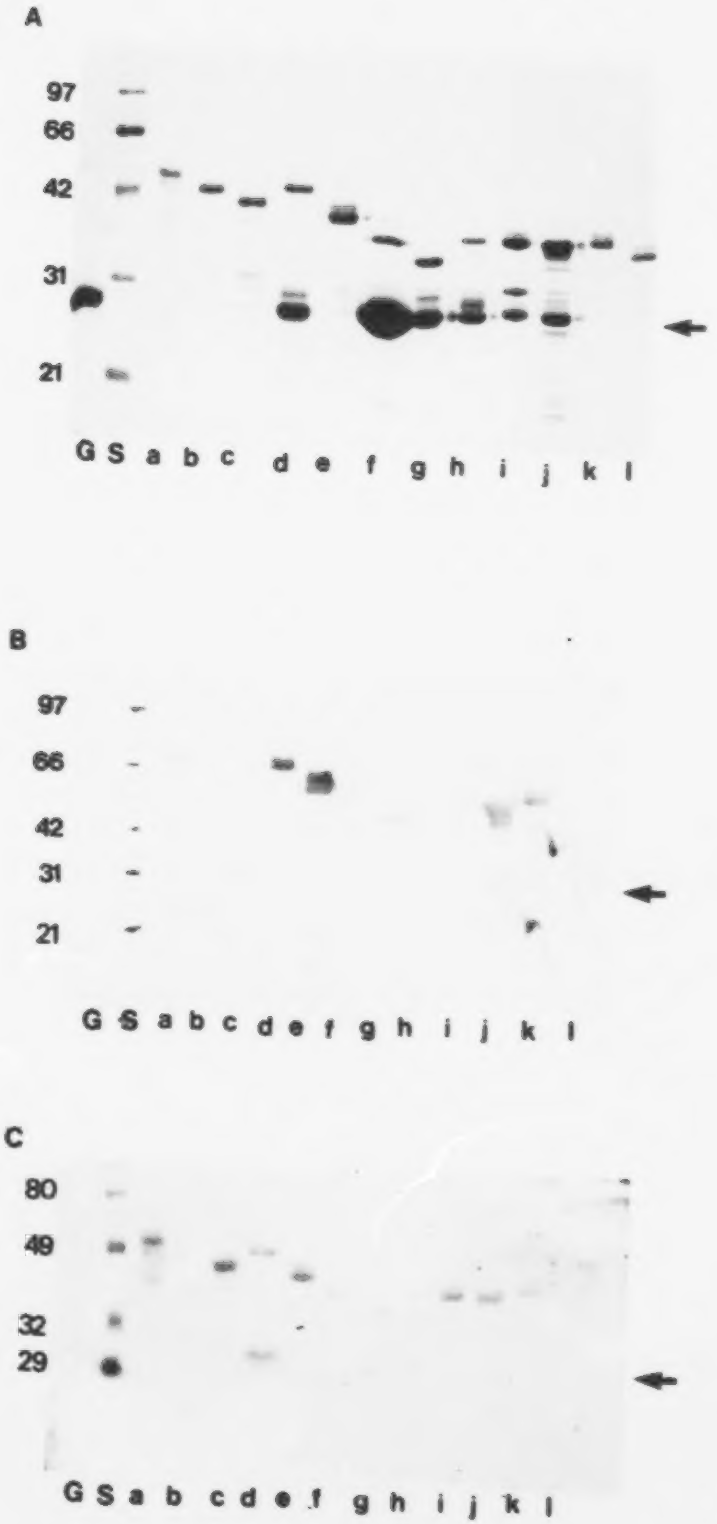

goat α-P-domain epitope
WNSEDDWDFLPPKKIKDPDASKPEDWDERAKIDDPTDSKPEDWDKPEHIPDPDAKKPEDWDEEMDGEWEPPVIQNPEYKGEWKPRQIDNPDYKGTWI*EF
180
Q --> K
273
HA1
185
230
HA3
185
199P --> T
204E --> K
250
HA5
185
191I --> V
233P --> T
SLEHVTD
245
HA8
185
191
PEDCDKPE
215K --> T 219W --> F
215
250
HA10
208
241
083
196
241
0811

Epitopic specificity of the goat anti-P-domain antibody

Figures 4-8B showed that the GST-P-domain (lane d), GST-HA1 (lane e), GST-HA3 (lane g), GST-HA8 (containing amino acid residues 185 - 245 and containing a 233P --> 233T mutation) (lane j), and GST-HA9 (containing amino acid residues 185 - 250 and containing a 217E ---> 217Q mutation) (lane k) reacted strongly with the goat anti-P-domain antisera. This antibody did not recognize the GST-C-domain (lane c) and the GST-N-domain (lane b). Weak reactivity was observed to GST-HA5 (containing amino acid residues 185 - 250 and containing 199P --> 199T and 204E-->K mutations within the first KPEDWDE sequence) (lane g), GST-HA6 (containing amino acid residues 185 - 253 and containing 4 inserted amino acids at position 191: IK --> ASRTE) (lane h), GST-HA10 (missing the first repeat sequence A) (lane l) and synthetic peptide 083 (missing the first repeat sequence A). GST-HA2 (lane f) also showed weak reactivity. This sample was similar to GST-HA8 (lane j) but does not contain the mutation. DNA sequencing of the plasmid containing the GST-HA2 construct confirmed that GST-HA2 had the same sequence as the GST-HA8 sample. We were unable to explain the lack of reactivity of the GST-HA2 sample with the goat anti-P-domain antibody. It is likely that the sample used for goat anti-P-domain antibody reaction was degraded. We have observed that most of the truncated GST-P-domain fusion proteins were sensitive to degradation despite the presence of protease inhibitors (see Fig. 4-8A - position of arrow denotes mobility of GST). Based on these results, the goat anti-P-domain antibody did not recognize a sample missing or having a changed first repeat sequence A (peptide 083; GST-N-domain, the GST-C-domain, GST-HA10, GST-HA5,

Figure 4-8: Identification and characterization of the truncated GST-P-domain fusion proteins

Truncated GST-P-domain fusion proteins were expressed in E. coli. and purified on a Glutathione-Sepharose affinity column and then separated by SDS-PAGE (12.5 % gel) (A), transferred electrophoretically to nitrocellulose membranes and incubated with goat anti-P-domain antibody (B), or incubated with $^{45}Ca^{2+}$ (C) as described under "Chapter Two: Experimental Procedures.". Lane a, purified pancreatic calreticulin; lane G, Recombinant GST; lane b, GST-N-domain; lane c, GST-C-domain; lane d, GST-P-domain; lane e, GST-HA1; lane f, GST-HA2; lane g, GST-HA3; lane h, GST-HA5; lane i, GST-HA6 ; lane j, GST-HA8; lane k, GST-HA9; lane l, GST-HA10. S, Bio-Rad low molecular weight standards in A, B, and C as indicated. The appearance of the Mr standards in C is due to the light emission from the dye bound to the standards proteins. The position of GST is indicated by the arrow.


A
97
66
42
31
21
G S a b c d e f g h i j k l
B
97
66
42
31
21
G S a b c d e f g h i j k l
C
80
49
32
29
G S a b c d e f g h i j k l

and GST-HA6), but recognized a sample containing a wild type first repeat sequence A - calreticulin, GST-P-domain, and GST-HA3. Antibody reaction of GST-HA3 (lane g) (containing the first two repeat sequence A segments) with the goat anti-P-domain antibody is stronger than GST-HA10 (lane l) (containing the last two repeat sequence A segments), but weaker than GST-P-domain (lane d) or calreticulin (lane a) (both containing all three repeat sequence A segments). We can therefore conclude that the first and third repeat sequence A segments may be required for antigenic recognition with this antibody, and that the epitopic site may be localized towards the first repeat sequence A. Therefore, a possible location of the epitopic site of this antibody may be within the first repeat sequence A of the P-domain, corresponding to residues 185 - 208 (Fig. 4-7) (since these residues were missing in the sample that showed weak reactivity to the antibody - GST-HA10).

Localization of the high affinity Ca^{2+} binding site within the P-domain

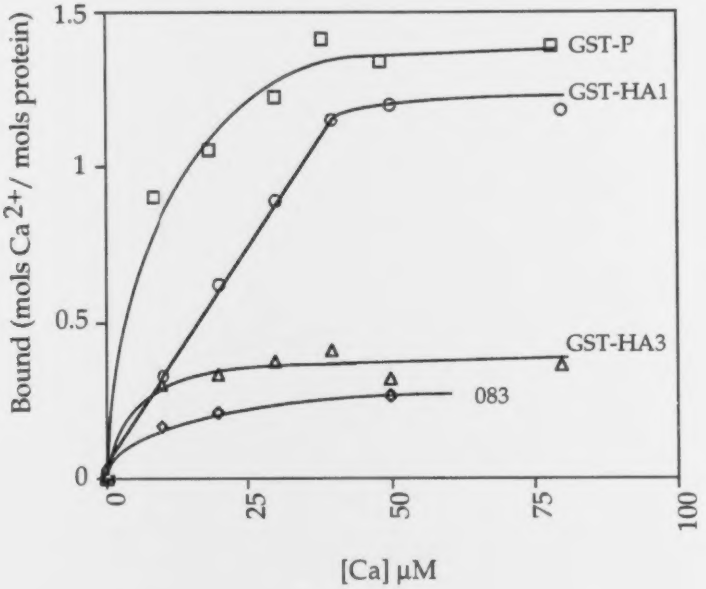
Ca^{2+} binding using the $^{45}Ca^{2+}$ overlay technique indicated strong binding to native calreticulin (Fig. 4-8C, lane a), the GST-C-domain (lane c), the GST-P-domain (lane d), GST-HA1 (lane e), GST-HA6 (lane i), GST-HA8 (238E-->S mutation) (lane j), and GST-HA9 (217E-->Q mutation) (lane k). In agreement with earlier observations, no Ca^{2+} binding was observed to the GST-N-domain (lane b). Only weak binding of $^{45}Ca^{2+}$ was observed for GST-HA2 (lane f), GST-HA3 (lane g), GST-HA5 (lane h), and GST-HA10 (lane l) fusion constructs. The GST-HA3 fusion protein was missing the third repeat sequence A of the P-domain, whereas both GST-HA10 and peptide 083 were missing the first repeat sequence A.

GST-HA5 was similar to GST-HA8 in length and contained 199P --> 199T, 204E --> 204K, and 237D -->G mutations. GST-HA2 (lane g) showed weak binding as determined by the $^{45}Ca^{2+}$ technique. As mentioned before, this sample had an identical sequence to GST-HA8, which was found to bind Ca^{2+}. This apparent discrepancy could not be explained. Similar to the sample used in the goat anti-P-domain antibody reaction, it is likely that the sample used for $^{45}Ca^{2+}$ overlay analysis was also degraded. *Correction:* After the thesis was submitted, analysis of the GST-HA5 and GST-HA10 samples used in the $^{45}Ca^{2+}$ overlay analysis revealed that both of these samples had degraded to the level of GST and smaller bands. The P-domain of calreticulin appears to be very susceptible to degradation. Analysis of the intact GST-HA5 and GST-HA10 samples revealed that they did bind Ca^{2+} using the $^{45}Ca^{2+}$ overlay and the Ca^{2+} microelectrode methodologies. Furthermore, Ca^{2+} binding analysis (via the $^{45}Ca^{2+}$ overlay technique) of a sample with a mutation within the first repeat sequence A (195D --> 195A) revealed only 30% binding using the $^{45}Ca^{2+}$ overlay analysis; whereas a 193D --> 193A mutation did not result in a reduction in the Ca^{2+} binding to this domain. The latter two samples were obtained after the thesis was submitted. These observations illustrate the importance of residues within the first and last repeat sequence A in high affinity Ca^{2+} binding to calreticulin's P-domain. As previously shown, the synthetic peptide 0811 (containing all three repeat sequences A) showed increased $^{45}Ca^{2+}$ binding over peptide 083 that was missing the first repeat sequence A (Fig. 4-5C). The former peptide, however, failed to bind Ca^{2+} when measured by the equilibrium dialysis technique. Therefore, in addition to residues within the first and last

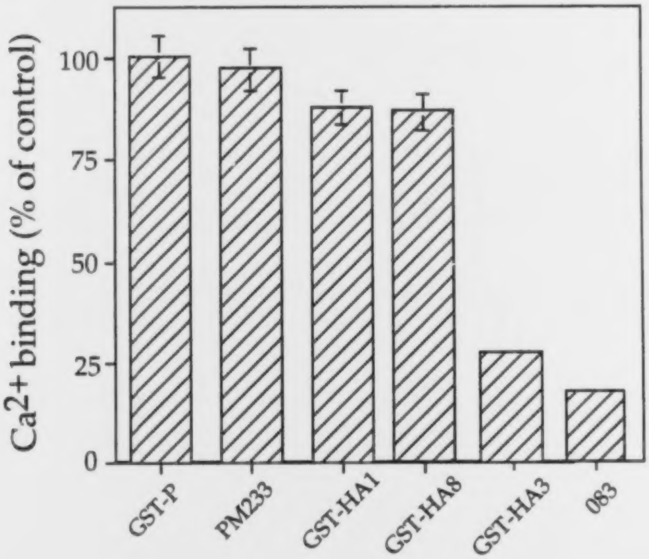
Figure 4-9: Ca^{2+} binding to the truncated GST-P-domain fusion proteins

Ca^{2+} binding to GST-fusion proteins was carried out using the Ca^{2+} microelectrode using a range of Ca^{2+} concentrations from 0.5 nM to 500 μM as described under "Chapter Two: Experimental Procedures." (A) Saturation curves for the binding of Ca^{2+} to GST-HA1, GST-P, GST-HA3, and peptide 083. SDS-PAGE of these samples is shown in Figure 4-6A. A binding curve similar to GST-HA1 was obtained for GST-HA8 and was omitted for simplicity. (B) Bar graph comparing the B_{max} values for the samples in (A) with the addition of PM233 (Factor Xa proteolytic P-domain fragment - see Fig. 4-5A) and GST-HA8. Ca^{2+} binding to the GST-P-domain was used as 100% value for Ca^{2+} binding.

A


Bound (mols Ca $^{2+}$/ mols protein)
1.5
1
0.5
0
0
25
50
75
100
[Ca] μM
GST-P
GST-HA1
GST-HA3
083

B


Ca^{2+} binding (% of control)
100
75
50
25
0
GST-P
PM233
GST-HA1
GST-HA8
GST-HA3
083

repeat sequence A within the P-domain, flanking amino acid contacts may be necessary to form the high affinity Ca^{2+} binding site in the peptide 0811, with residues within the first and the last repeat sequence A being the most critical. The observed quenching of the aromatic fluorescence observed in Fig. 4-6 (inset) could represent movement of the first and third repeats, containing only an aromatic tryptophan residue, to create the high affinity binding site for the Ca^{2+}. Changes within the second repeat sequence may not have occurred upon Ca^{2+} binding and thus may account for the population of tryptophan residues that did not undergo a change in ellipticity when Ca^{2+} was added (positive bands at 297 nm and 287 nm).

Ca^{2+} binding to the GST-fusion proteins and synthetic peptides was further analyzed using a Ca^{2+} microelectrode (Fig. 4-9). Based on the $^{45}Ca^{2+}$ overlay results, truncated GST-P-domain fusion proteins GST-HA1, GST-HA3, GST-HA8, and GST-HA10 were selected for quantitative Ca^{2+} binding analysis using the Ca^{2+} microelectrode. This method allowed measurement of Ca^{2+} concentrations from 10^{-6} to 10^{-4} M. This was in the range of high affinity Ca^{2+} binding to calreticulin (K_d of 4 - 10 μM). Saturable binding ability was obtained for the GST-P-domain, GST-HA1, and GST-HA8 fusion proteins, binding 1.34, 1.2, and 1.1 mole of Ca^{2+}/mole of protein, respectively (Fig. 4-9A). All three of these fusion proteins thus bound Ca^{2+} with affinities comparable to the high affinity Ca^{2+} site within native calreticulin (Table 4-2). However, peptide 083 (having the last two repeat sequence A segments) bound only 0.265 mole Ca^{2+}/mole of protein, whereas binding increased to 0.369 mole Ca^{2+}/mole for the truncated GST-P-domain fusion protein GST-HA3

Table 4-2:

Summary of the characterization of the truncated GST-P-domain fusion proteins

	His-CRT *1	GST-P	PM233	GST-HA1	GST-HA8	GST-HA3	GST-HA10	0811	083	GST
#aa	401	130	96	98	60	49	49	45	33	225
MW *2	46 567	42 103	10 505	37 750	34 057	32 263	31 902	5 334	3 480	26 267
pI *3	4.13	4.06	4.06	4.04	4.07	4.15	3.86	3.92	3.89	5.872
goat α–P-domain reactivity	++	++++	+++	++++	+++	++	-	ND	-	-
$^{45}Ca^{2+}$ overlay	++++	++++	+++	++++	+++	++	+++	++	+	-
Ca binding *4 K_d	7 μM	6 μM	10 μM	7 μM	9.8 μM	4 μM	102 μM	ND	ND	-
B_{max}	1.9	1.34	1.3	1.17	1.1	0.36	0.84	ND	0.239	-
% D/E	27	29	31	32	31	30	34	40	36	16

Note: "-" denotes lack of reactivity with the goat anti-P-domain antibody or lack of $^{45}Ca^{2+}$ binding as determined using the $^{45}Ca^{2+}$ overlay technique. "+" denotes reactivity with the goat anti-P-domain antibody or $^{45}Ca^{2+}$ binding as determined using the $^{45}Ca^{2+}$ overlay technique. "ND" - -not determined; "# aa" - number of amino acids; "% D/E" - percentage of aspartic (D) and glutamic (E) amino acids.

*1 Histidine-tagged calreticulin

*2 Molecular weight obtained from cDNA encoding the fusion protein (GST M_r included)

*3 pI value reflects truncated P-domain portion of the fusion protein

*4 Ca^{2+} binding determined using a Ca^{2+} microelectrode. Values indicated for k_d and B_{max} were obtained from duplicate experiments using 300 μg of protein. See "Chapter Two: Experimental Procedures" for details on experimental design.

(having the first two repeat sequence A segments). GST-HA10 (which was missing the first repeat sequence A) bound 0.84 mole Ca^{2+}/mole of protein with a K_d of 102 µM. Comparison of the B_{max} values for Ca^{2+} binding to these proteins (Fig. 4-9B) further supported the importance of residues within the first and third repeat sequence A within the P-domain of calreticulin in high affinity Ca^{2+} binding. When all three repeat sequence A segments were present (in the GST-P-domain, GST-HA1, and the GST-HA8 fusion proteins; and in the truncated P-domain fragment PM233), we observed close to optimal Ca^{2+} binding. When the first repeat sequence A was missing (peptide 083 and GST-HA10), binding capacity was reduced to 18% Ca^{2+} binding capacity and in the case of GST-HA10, the K_d was 10X that of the wild type indicating that the coordination of the Ca^{2+} ion (in this sample) may be to residues that have a weaker coordination strength than the residues within the first repeat sequence A. When the last repeat sequence A was missing (GST-HA3), binding capacity was reduced to a level of 27% of maximal (Fig. 4-9B). Summarizing the results in Table 4-2, it is apparent that having negative charges within the protein sequence was important but not sufficient for high affinity Ca^{2+} binding. GST-HA3 has a %D/E content comparable to GST-HA1, GST-HA8 and GST-P-domain (30%, 31%, 32%, and 29%, respectively), but only had 27% Ca^{2+} binding capacity. Also the pI values of GST-P-domain, GST-HA1, GST-HA8, and GST-HA3 were similar (4.06, 4.04, 4.07 and 4.07, respectively). Peptide 083 was more acidic than all of the other fusion protein having a %D/E content of 36%, but having only 18% Ca^{2+} binding capacity. Therefore, deletion of one of first or third repeat sequence A did not change the acidic content of the protein but appeared to destroy or weaken the high affinity Ca^{2+} binding structure within the P-domain of calreticulin (compare B_{max} of GST-HA3 and 083 to GST-P-domain and GST-HA1 samples, Fig. 4-9B; and the K_d of GST-HA1 and GST-HA10 in Table 4-2). These results thus support the importance of residues within the first and third repeat sequence A in high affinity Ca^{2+} binding, with the last repeat sequence A possibly providing most of the Ca^{2+} chelating residues in the coordination sphere for Ca^{2+}. The binding of Ca^{2+} to both GST-HA1 (98 amino acids long) and GST-HA8 (60 amino acids long) and the lack of binding to 083 (33 amino acids long) would thus indicate that the high affinity Ca^{2+} binding site within calreticulin's P-domain can be localized to a segment greater than 33 amino acids (between residues 208 - 241) and shorter than 60 amino acids (between residues 185 - 245).

DISCUSSION

In this chapter we showed that both native and recombinant calreticulin bound Ca^{2+} with high and low affinities. We have further confirmed our earlier suggestion that the high capacity, low affinity Ca^{2+} binding site was present in the carboxyl-terminal domain of calreticulin (Fig. 4-10). Importantly, we have also specifically localized the high affinity Ca^{2+} binding site to the P-domain of calreticulin (Fig. 4-10). Since recombinant GST did not bind Ca^{2+}, by either equilibrium dialysis or $^{45}Ca^{2+}$ overlay and did not stain blue with Stains-All. Therefore, it did not interfere with the identification and characterization of recombinant Ca^{2+} binding proteins (see Fig. 3-3F, lane 2). The GST fusion protein system is therefore an excellent means by which to study the structure

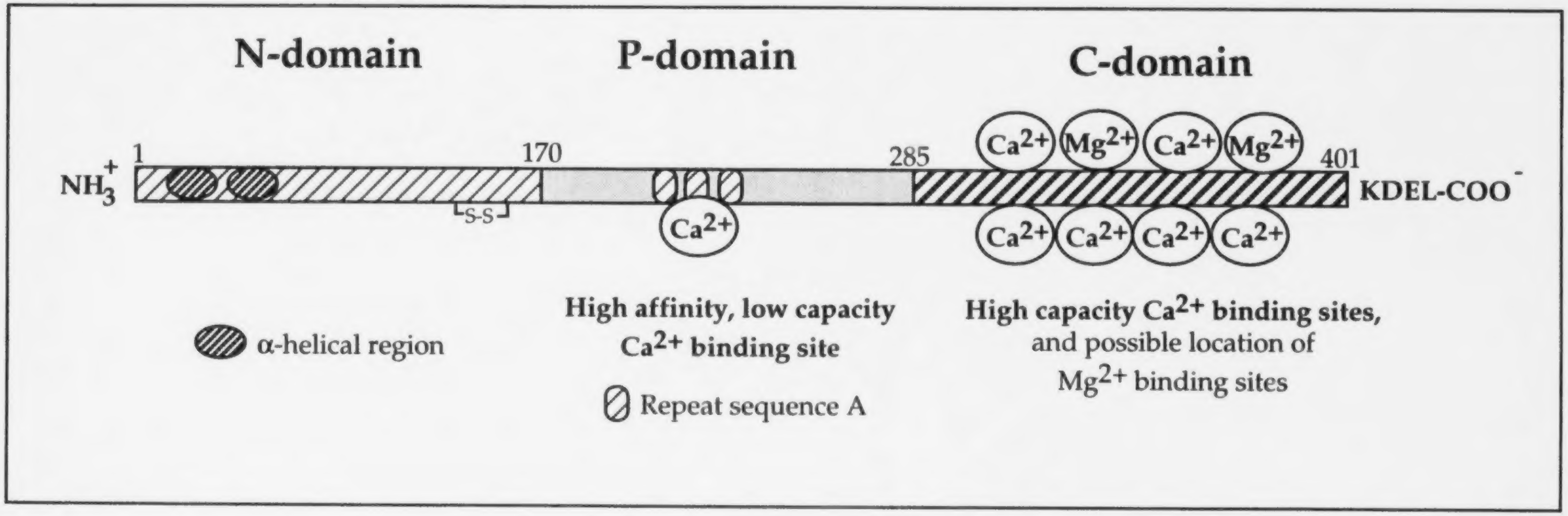

N-domain
P-domain
C-domain
NH$_3^+$
1
170
285
401
KDEL-COO$^-$
S-S
Ca^{2+}
Ca^{2+}
Mg^{2+}
Ca^{2+}
Mg^{2+}
Ca^{2+}
Ca^{2+}
Ca^{2+}
Ca^{2+}
α-helical region
High affinity, low capacity Ca^{2+} binding site
Repeat sequence A
High capacity Ca^{2+} binding sites, and possible location of Mg^{2+} binding sites

Figure 4-10: Calreticulin domains involved in Ca^{2+} binding

Putative location of the high affinity, low capacity Ca^{2+} binding site (P-domain) and of the low affinity, high capacity Ca^{2+} binding sites (C-domain) in calreticulin are indicated. Location of the Mg^{2+} sites are also indicated (within the C-domain). Numbers indicate amino acid position within rabbit skeletal muscle calreticulin sequence.

and function of Ca^{2+} binding proteins. Native calreticulin bound 1 mole of Ca^{2+} per mole of protein with high affinity, and approximately 20 moles of Ca^{2+} per mole of protein with low affinity (Ostwald and MacLennan, 1974). We have showed that recombinant calreticulin and the native protein have virtually identical Ca^{2+} binding properties.

Both high and low affinity Ca^{2+} binding sites had been previously described in native calreticulin (Ostwald and MacLennan, 1974). However, Waisman *et al.* (1987) and Van *et al.* (1989) reported only high affinity Ca^{2+} binding to calreticulin, whereas Macer and Koch (1988) and Treves *et al.* (1990) showed only low affinity Ca^{2+} binding to this protein. These discrepancies were most likely explained by the different methods used for measurement of Ca^{2+} binding by these authors. Macer and Koch (1988) and Treves *et al.* (1990) studied Ca^{2+} binding to calreticulin only at relatively high concentrations of Ca^{2+}, and consequently did not detect any high affinity Ca^{2+} binding. Waisman *et al.* (1987) and Van *et al.*, (1989) studied Ca^{2+} binding to calreticulin in the presence of millimolar concentrations of Mg^{2+}, which we found to reduce Ca^{2+} binding to the high capacity, low affinity sites by 60%. The studies reported here show that calreticulin does in fact bind Ca^{2+} with both a low and a high affinity, and that these binding properties represent distinct Ca^{2+} binding sites which are located in different domains of the protein. Interestingly, Mg^{2+} competes with Ca^{2+} for the low affinity high capacity binding site. The physiological significance of this competition has yet to be determined. Baumann *et al.* (1991) recently studied the ion content of the ER membranes in honey bee photoreceptors using electron X-ray microanalysis. They found that light stimulation caused a significant increase in the Mg^{2+} content of the ER membranes, and they proposed that Mg^{2+} could balance charge movement related to Ca^{2+} release from ER membranes. Since Mg^{2+} competed with Ca^{2+} for binding sites on calreticulin it is possible that Mg^{2+} taken into ER membranes could be "stored" bound to calreticulin.

In order to define the location of the two types of Ca^{2+} binding site in calreticulin we expressed different domains of the protein, using the GST fusion protein system. Using a $^{45}Ca^{2+}$ overlay technique we discovered that the C- and P-domains bind Ca^{2+} whereas the N-domain did not. It is possible that the N-domain of calreticulin might bind Ca^{2+} but not after SDS-PAGE and transfer to nitrocellulose membrane. This is unlikely since the N+P-domain showed the same binding properties as the P-domain (Table 4-1). Therefore, the C- and P-domains must be responsible for Ca^{2+} binding to the whole protein. In addition we found that Ca^{2+} bound with low affinity and high capacity specifically to the C-domain of calreticulin. This result was not surprising, since this part of the protein consists of clusters of aspartic and glutamic acid residues. Similar clusters of acidic amino acid residues are found in the carboxyl-terminal region of skeletal and cardiac muscle calsequestrin (Fliegel *et al.*, 1987; Scott *et al.*, 1988), and Ohnishi and Reithmeier (1987) have isolated a carboxyl-terminal fragment (domain) of calsequestrin and shown that it is involved in the high capacity, low affinity Ca^{2+} binding to this protein. It is important to note that the capacity for low affinity Ca^{2+} binding is different in these two proteins (calsequestrin approximately 50 moles/mole of protein versus calreticulin approximately 17-21 moles/mole of protein), despite the fact that the number of acidic residues in both proteins is very similar. However, close examination of the acidic carboxyl-terminal regions of these two Ca^{2+} binding proteins revealed significant differences in the sequence. For example, while a majority of the last 50 amino acids in both proteins are highly acidic, in the case of the two isoforms of calsequestrin this region consisted of long, uninterrupted, stretches of aspartic and glutamic acid residues; in calreticulin the acidic residues in this region of the molecule are interspersed at regular intervals with one or more positively charged residues. It was shown by Breier and Michalak (1994), using the amino group modifier 2,4,6-trinitrobenzenesulfonic acid (TNBS), that the amino-groups of the positively charged lysine residues in the C-domain were important for high capacity, low affinity Ca^{2+} binding within this domain of calreticulin. This difference might lead to different folding patterns for this part of the molecule for calreticulin compared with calsequestrin and would probably affect the net charge of this region, resulting in the differing capacity for Ca^{2+} binding.

Several other Ca^{2+} binding proteins are now known to have clusters of acidic amino acid residues, suggesting that these residues might be involved in the observed Ca^{2+} binding. For example, chromogranin A, a Ca^{2+} binding protein found in the chromaffin granules in the variety of endocrine and neuronal cells (Simon and Aunis, 1989), binds large amounts of Ca^{2+} (Yoo and Albanesi, 1991) and contains clusters of acidic amino acid repeats (Helman *et al.*, 1988). The ryanodine receptor/Ca^{2+} channel of SR membranes also contains clusters of acidic residues (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990), and the closing of this Ca^{2+} channel is sensitive to millimolar Mg^{2+} and/or Ca^{2+}

concentrations (Meissner, 1986). The cytoplasmic part of the molecule (amino acid residues 1842 to 1938) is particularly enriched in glutamic and aspartic acid residues (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990), and, therefore, may be involved in the binding of Ca^{2+} and/or Mg^{2+}. It is tempting to speculate that this binding site may be involved in Ca^{2+} and/or Mg^{2+} binding and perhaps in the response of the channel to changes in cytoplasmic Ca^{2+} concentrations. A number of other proteins have been shown to have one or more clusters of acidic residues, including nuclear histone-binding protein N1/N2 (Kleinschmidt *et al.*, 1986), HMG-1 chromatin protein (Walker *et al.*, 1978), nucleolin (Lapeyre *et al.*, 1987), and the centromere specific protein, CENP-B (Earnshaw *et al.*, 1987). The acidic residues in these proteins have been implicated to play a key role in the possible interactions of these molecules with other proteins. As yet the role of Ca^{2+} binding, if any, in these regions has not been determined.

In a search for homology between calreticulin and other known proteins, we noted amino acid identities (similarities) between the C-domain of calreticulin and a number of other proteins, including PDI, BiP, GRP96 and some heat shock proteins (Fliegel *et al.*, 1989c). All of these proteins, including calreticulin, terminate with the KDEL ER retention signal proceeded by clusters of acidic residues. It has been proposed that Ca^{2+} might be involved in the regulation of the interactions between KDEL proteins and the KDEL receptor (Kelly, 1990). Ca^{2+} bound to the C-domain of these proteins may, therefore, be involved in these interactions. This suggestion was supported by the observation that addition of the KDEL sequence to proteins without upstream acidic residues upstream lead to only partial retention of these molecules within the lumen of the ER (Zagouras and Rose, 1989; Sonnichsen *et al.*, 1994). The C-domain of calreticulin and other KDEL proteins may, therefore, have a dual function: (i) Ca^{2+} storage and (ii) control of the retention of the protein in the lumen of the ER.

The most important finding of this study was the observation that the high affinity Ca^{2+} binding site of calreticulin was located in the P-domain. This domain is characterized by a high number of proline residues (16 mole %) which are spaced every 4 or 5 amino acids. This sequence was predicted to contain a repeating, rigid turn structure, which separated the globular head of the protein (N-domain) from the acidic tail (C-domain). The P-domain also contained a relative concentration of aspartic acid (24 mole %), glutamic acid (15 mole %) and tryptophan (7 mole %) residues. The P-domain contained the repeat sequence A (PXXIXDPDAXKPEDWDE), which was repeated three times, and followed by a proline-, serine- and threonine-rich sequence (residues 247 - 317), which was predicted to have a high turn/loop potential. Ca^{2+} binding to the P-domain was demonstrated by $^{45}Ca^{2+}$ overlay and was quantified by equilibrium dialysis. This domain bound approximately 1 mole of Ca^{2+}/mole of protein with a dissociation constant of 6-11 µM. Other proteins that bind Ca^{2+} with similar selectivity and affinity are characterized by the presence of a common structural motif, the EF-hand (Kretsinger *et al.*, 1988). The EF-hand is a characterized by a linear sequence of ~ 30 amino acids where two nearly perpendicular α-helices flanking a 12-residue Ca^{2+} chelating domain (Kretsinger *et al.*, 1988). The consensus 12-residue Ca^{2+} chelating domain is shown below and is compared to the three repeat sequence A segments within calreticulin's P-domain (denoted by A-1, A-2, and A-3):

Coordination sphere	X		Y		Z		-Y		-X			-Z
CaM	D	A/K	D	G	D/N	G	T/Y/Q	I	T/D/S	T/F/A	K/P	E
CRT A-1	^{193}D	P	D	A	S	K	P	E	D	W	D	E
A-2	**^{210}D**	**P**	**T**	**D**	**S**	**K**	**P**	**E**	**D**	**W**	**D**	**K**
A-3	^{227}D	P	D	A	K	K	P	E	D	W	D	E

Residues forming the Ca^{2+} coordination sphere (denoted X, Y, Z, -Y, and -Z) are underlined. These Ca^{2+} chelating residues are usually negatively charged or contain oxygen and/or nitrogen atoms. The coordination sphere is completed by a water molecule in position -X (Beckingham, 1991; Starovasnik *et al.*, 1992). Each Ca^{2+} ion is coordinated by seven oxygen ligands in a pentagonal bipyramidal configuration; five of the oxygens are provided by side chains (positions X, Y, Z, and -Z, the residue at position -Z contributing two oxygens), one by a backbone carbonyl (at position -Y) and one by a water molecule (at position -X). In many EF-hand structures, only 4 or 5 of the potential chelating residues are used to bind Ca^{2+} (Strynadka and James, 1989).

Analysis of the amino acid sequence of calreticulin did reveal a potential to form the EF-hand sequence. If A-1 and A-3 form the helices, then A-2 would be predicted to form the Ca^{2+} chelating 12-residue loop (shown in bold). This loop contained identical position X residues; a conserved position Z (D/N --> S; polar side chain conservation);

hydrogen bonding residue at position -X (D); position -Y requires only a backbone carbonyl which could be provided by proline (P). Position Y does not have the aspartic acid to provide the electrostatic interaction with the Ca^{2+} ion, but this could be provided by the other two repeat segments (A-1 and A-3) when they come together to trap the Ca^{2+} ion. Position - Z is also different, but as determined by Strynadka and James (1989), not all of the chelating residues may be involved in Ca^{2+} coordination. It is therefore possible that the P-domain may contain the helix-loop-helix secondary structure associated with high affinity binding of Ca^{2+} (Fig. 4-5B and above - A-1 forming the first helix; A-2 forming the Ca^{2+} chelating loop; and A-3 forming the last helix). We would then predict that all three repeat segments would therefore be required for high affinity Ca^{2+} binding to calreticulin. Analysis of the Ca^{2+} binding to the synthetic peptides and to the truncated P-domain proteins revealed a requirement for residues within the first and third repeat sequence A in high affinity Ca^{2+} binding to the P-domain. Ca^{2+} binding, as measured by the $^{45}Ca^{2+}$ overlay technique and Ca^{2+} microelectrode, was reduced when residues within the first or third repeat sequence A were absent (GST-HA3 and GST-HA10). A truncated GST-P-domain fusion protein that contained a 195D-->A (in repeat sequence A-1) was found to bind very little Ca^{2+} as determined by the $^{45}Ca^{2+}$ overlay technique, whereas a truncated GST-P-domain fusion protein that contained a 193D-->A (in repeat sequence A-1), 204E-->K (in repeat sequence A-1 - sample GST-HA5), 217E-->Q (in repeat sequence A-2 - sample GST-HA9), 237D-->G (in repeat sequence A-3 - sample GST-HA5), and 238E-->S (in repeat sequence A-31 - sample GST-HA8) was found to bind Ca^{2+} at a level comparable to the wild type. The 195D-->A mutation is located at position Y of A-1 and may be important for providing a chelating residue for Ca^{2+} coordination. Therefore, as observed, a mutation at this position disrupted the coordination of the Ca^{2+} ion. The other mutations apparently did not influence the Ca^{2+} binding ability of the P-domain. If the entire first repeat sequence A was directly involved in chelating the Ca^{2+} ion, then incubation of a P-domain sample with the goat anti-P-domain antibody (the epitope corresponding to residues 185 - 208) should result in a drastic reduction in the Ca^{2+} binding ability of that protein. When the GST-HA1 fusion protein was incubated with the goat anti-P-domain antibody, followed by dot blotting onto nitrocellulose membrane and incubation with $^{45}Ca^{2+}$, no reduction in the $^{45}Ca^{2+}$ binding ability of GST-HA1 was observed. Therefore, it appeared that only certain residues within the first repeat sequence A may be involved in the Ca^{2+} chelating loop (one residue being the aspartic residue at position 195). Most of the Ca^{2+} chelating residues may be originating from repeat sequence A-3 (which is missing in GST-HA3). These observations thus lend support to the importance of residues within the first and third repeat in the formation of the EF-hand-like high affinity Ca^{2+} binding site within calreticulin's P-domain. It would be curious to determine if removal/replacement of the potential Ca^{2+} chelating loop (A-2) would destroy the binding of 1 mol of Ca^{2+} to the P-domain.

Two ER proteins were shown to contain calreticulin's P-domain repeat sequences and bind $^{45}Ca^{2+}$ using the $^{45}Ca^{2+}$ overlay methodology - calmegin/calnexin-t, an abundant male germ cell-specific Ca^{2+} binding protein of the ER (with 60% amino acid identity to mouse calnexin)

(Ohsako *et al.*, 1994; Watanabe *et al.*, 1994), and calnexin, an ER transmembrane chaperone (Tjoelker *et al.*, 1994). Both of these proteins contained four repeat sequence A repeat sequences, three of which are identical to calreticulin's (Fig. 4-11A). Using the $^{45}Ca^{2+}$ overlay methodology, Tjoelker *et al.* (1994) showed that an 80 amino acid sequence containing the repeats (amino acid 254 - 334 of calnexin) was sufficient to bind Ca^{2+} with high affinity. Tjoelker *et al.* (1994) compared the proline rich domains of several calnexin species and two different calreticulins (Fig. 4-11B). The calnexin family required four repeat sequence A segments for high affinity Ca^{2+} binding whereas the calreticulin family only required three repeat sequence A segments for high affinity Ca^{2+} binding. Tjoelker *et al* (1994) also showed that the presence of a second repeat sequence ("column B" in Fig. 4-11B and underlined sequences in Fig. 4-11A) which they determined not to be directly essential for high affinity Ca^{2+} binding, but may provide a possible structural role in the high affinity Ca^{2+} binding site. The importance of this second repeat sequence still remains to be tested in calreticulin.

Recently, a single gene has been isolated from yeast *Saccharomyces cerevisiae*, CNE1, that was found to share some of the motifs with calnexin and calreticulin (Parlati *et al.*, 1995). Similarity at the amino acid level was approximately 31% to calnexin and 24% to calreticulin. The function of this gene product still has to be determined, but similarities with calnexin and calreticulin may suggest a potential chaperone-like function for the CNE1 gene product, assisting in the recognition and retention of some mutant proteins and components of unassembled

Figure 4-11: Amino acid comparison between the P-domain of rabbit calreticulin and rabbit calnexin

(A) Amino acid sequence of canine calnexin and rabbit calreticulin were taken from Wada *et al.* (1991) and Fliegel *et al.* (1989b), respectively. "*" denotes highly conserved amino acid positions; ":" denote amino acid identity; "-" denotes amino acid gaps in the sequence of calreticulin; repeat sequence A is labeled A-1, A-2, A-3; underlined segments after repeat A-3 are part of column B (see below). Underlined sequence before repeat A-1 represents the nuclear localization signal in calreticulin. Numbers on top represent calnexin amino acid positions and numbers on bottom represents calreticulin amino acid positions.

(B) Column A depicts the first consensus repeat sequence (repeat sequence A) which binds calcium (calnexin residues 253 - 324 and calreticulin residues 172 - 250). Column B represents a second consensus repeat sequence (repeat sequence B) which does not bind calcium (calnexin residues 328 - 388, and calreticulin residues 241 - 280). Sequences are listed as they appear from amino to carboxyl termini of the proteins. Consensus residues are defined as those present in more than 50% of the aligned sequences. Residues appearing more than 90% of the sequences are indicated (*). Periods represent identity to the consensus residues. Reproduced from Tjoelker *et al*, 1994.

A

```
                                                         Repeat A-1           Repeat A-2
               260                                       **    *      * *     **    *  310
Canine CNX   ILVDQSIVNS GNLLNDMTPP VNPSREIEDP EDQKPEDWDE RPKIPDPDAV KPDDWNEDAP
Rabbit CRT   VKI:N:QVE: :SLED:WD-- FL:PKK:K:: DAS::::::: :A::D::TDS ::E::D----
               172                                                         202

                         Repeat A-4        .. Repeat A-3
           ..       320                          **    *        *      **        *
Canine CNX   AKIPDEEATK PDGWLDDEPE YVPDPDAEKP EDWDEDMDGE WEAPQIANPK CESAPGCGVW
Rabbit CRT   ---------- -------:K:: HI:::::K:: ::::::E::: : ::P:V:Q::E ------YK:E
                ...                                                   250

                 **       *        **  395
Canine CNX   QRPMIDNPNY KGKWKPPMID NPNYQ
Rabbit CRT   KPRQ::::D: ::T:IH:E:: ::E:S
                       273
```

Calnexin sequence based on Tjoelker *et al* (1994); calreticulin sequence based on Fliegel *et al*. (1989a).

Top numbers indicate amino acid position within the calnexin sequence; bottom numbers indicate amino acid position within the calreticulin sequence. Position of calreticulin's Repeat sequence A is indicated by A-1, A-2, and A-3; the sequence comparison of these repeats is shown in below. Underlined sequence correspond to repeat sequence B (see below). Calnexin's fourth repeat sequence A is shown in bold between calnexin residues 310 - 326.

B

		A	B
		* * ** *	* * **
Consensus		PXXIPDPXAXKPEDWDE	GXWXPPXIXNPXYX
Human Calnexin		SRE.E..EDR.......	.E.EA.Q.A..RCE
		RPK....E.V..D....	.V.QR.V.D..N.K
		.AK...EE.T...G.LD	.K.K..M.D..S.Q
		.EYV...D.E.......	.I.K.RK.P..DFF
A.thaliana CNX1p		AKT....EDK.......	.M.EA.K.D..KCE
		RAK....N.V.......	.E.KR.MER..A.K
		.ME.E.EE.E...G.LD	.K.SS.L.D..A.K
		.EEVD..E.T......D	.I.K.RD.P..D.F
S. mansoni SmIrV1		.KE.D..EDK..S....	.E.VA.Q.N..KCA
		REK.V.TN.K..D....	.K.HR.I.P..K.K
		.AT.E.ES.V..SG.LD	.K.SA.M.P..N.K
		.EM....A.VP.K...R	.I.T.RK.P..H.F
Murine Calreticulin	1	.KK.K..D.A.......	.E.E..V.Q..E.K
	2	RAK.D..TDS......K	.E.K.RQ.D..D.K
	3	.EH....D.K.......	.T.IH.E.D..E.S
O. volvulus λRal-1		.KK.K..D.K.......	.E.E..MVD..E.K
		REF.D.EDDK......K	.E.K.KQKK..A.K
		.EH....D.K......D	.K.IH.E.EI.D.T
S. cerevisiae CNX Hom.		.LM...VSVA..H...D	.E.I..M.K..LCT
		RIR....E.V.LS.R..	.CQI.GL.N.AK.K
		.EY.L..N.Q..SW.K.	.E.H..E.E..L.Y

complexes (Parlati *et al.*, 1995). The CNE1 gene product, a 76 kDa glycosylated integral membrane ER protein, contained only one of calreticulin and calnexin's repeat sequence A segments (corresponding to the first repeat sequence A in calreticulin) and two of repeat sequence B segments (corresponding to the first two repeat sequence B in calreticulin) (Parlati *et al.*, 1995). Three other repeat sequence A-like segments were found in the CNE1 gene product, but they contained only 6/17 residues in the repeat sequence A motif (Fig. 4-11B) and was found to be missing key residues that are part of the EF-hand coordination sphere. The CNE1 gene product and a GST fusion of this gene product did not bind Ca^{2+} as determined by the $^{45}Ca^{2+}$ overlay technique (Parlati *et al.*, 1995). This supported our conclusion that more than one repeat sequence A was required for the high affinity Ca^{2+} binding to calreticulin. It is therefore possible that within the calreticulin family of proteins, three repeat sequence A segments are required for the high affinity binding of Ca^{2+} (with the first and last repeats being the most critical to Ca^{2+} binding), and that within the calnexin family, four repeat sequence A segments are required for the high affinity binding of Ca^{2+}.

Circular dichroism analysis on the GST-HA1 sample (free of GST) containing all three repeat sequence A and flanking regions revealed changes in the aromatic spectrum similar to those observed for the truncated P-domain fragment PM233 (Fig. 4-6). Both samples showed that upon Ca^{2+} binding, movement of two populations of tryptophan residues away from the surface is observed. Structural changes induced by the binding of Ca^{2+} are the result of a balance between the reorganization required for ion binding and the maintenance of contacts between the hydrophobic side chains in the interior of the protein (Skeldon *et al.*, 1994). Changes within the two populations of tryptophan residues could possibly indicate the reorganization and movement of the first and third repeat sequence A within the P-domain of calreticulin coming together to create the high affinity binding site binding 1 mole of Ca^{2+} / mole of protein. Preliminary 1D-NMR analysis on this sample, revealed very dramatic peak shifting that would indicate changes resulting upon the binding of Ca^{2+} (Sykes, Golden, Baksh, and Michalak, unpublished results). Two dimensional- and 3D-NMR analysis will allow peak assignments and a further understanding of the varied changes that Ca^{2+} induces when bound to its high affinity site within the P-domain of calreticulin.

Besides the EF-hand family of the high affinity Ca^{2+} binding proteins (Kretsinger *et al.*, 1988; Heizmann and Hunziker, 1991), a second group of Ca^{2+} binding proteins has been recognized, which belongs to the annexin family (binding 2 - 4 moles of Ca^{2+} / mole of protein and Ca^{2+} binding affinities ranging from 30 to 300 μM). Annexins are widely distributed proteins which bind phospholipids and cellular membranes in a Ca^{2+}-dependent manner (Klee, 1988; Burgoyne and Geisow, 1989; for terminology see Crumpton and Dedman, 1990; Raynal and Pollard, 1994). They are thought to be important in the control of cellular proliferation (Klee, 1988; Burgoyne and Geisow, 1989). The high affinity binding of Ca^{2+} to EF-hand proteins and/or annexins is usually associated with a regulatory functions and it is possible, therefore, that high affinity Ca^{2+} binding to the P-domain of calreticulin may also have some regulatory function. Based on amino acid sequence information from different calreticulins we conclude that calreticulin belongs to neither EF-hand nor annexin families of Ca^{2+} binding proteins, and that it may, therefore, belong to a third, yet to be recognized family of the high affinity Ca^{2+} binding proteins.

CHAPTER FIVE

Identification of the zinc binding sites within calreticulin

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INTRODUCTION

Zn^{2+} is under homeostatic regulation and plays an important regulatory role in intracellular signal transduction (Coleman, 1992; Hechtenberg and Beyersmann, 1993). The cytosolic $[Zn^{2+}]$ has been estimated at 200 to 500 μM in rat liver (Brand *et al.*, 1988). However, the concentration of Zn^{2+} may vary in the cytoplasm, ER and the nucleus. A number of proteins have been identified to bind Zn^{2+}. Most of these proteins are enzymes, transport-storage proteins, or proteins that interact with nucleic acid (Berg, 1990; Coleman, 1992). The regulatory role of Zn^{2+} is based on its activity in transcription factors and its binding to key enzymes involved in animal metabolism such as aspartate transcarbamoylase (Jefferson *et al.*, 1990) and parathymosin (Brand *et al.*, 1991), and to a key secondary stimulating enzyme protein kinase C (Quest et al., 1992). A number of Ca^{2+} binding proteins, including calmodulin, S100 (Baudier *et al.*, 1983; Baudier and Gerard, 1986) and calsequestrin (Ikemoto *et al.*, 1974) have been shown to bind Zn^{2+} suggesting that Zn^{2+} may also modulate their function.

Calreticulin is a major 60-kDa Ca^{2+} binding/storage protein of the ER (Michalak *et al.*, 1992). Recent evidence suggests that calreticulin (or calreticulin-like proteins) may also be found in the nucleus, nuclear envelope, the cytosol and on the cell surface (Michalak *et al.*, 1992). The protein has two classes of Ca^{2+} binding sites as described in the last chapter. Calreticulin also binds other ions including Mg^{2+} and Fe^{3+} (Conrad *et al.*, 1990 and 1993).

We are interested in the structural and functional analysis of the ER lumenal proteins and the role of ions in control of their functions. In the last chapter we have identified calreticulin as one of the major Ca^{2+} binding proteins of the ER membrane and localized the Ca^{2+} binding sites to different domains in the protein (Milner *et al.*, 1991; Baksh and Michalak, 1991). In this chapter we identified two distinct classes of Zn^{2+} binding sites in calreticulin. In addition, we have used the glutathione-S transferase (GST) fusion protein system to express distinct domains of calreticulin to identify region(s) of the protein involved in Zn^{2+} binding.

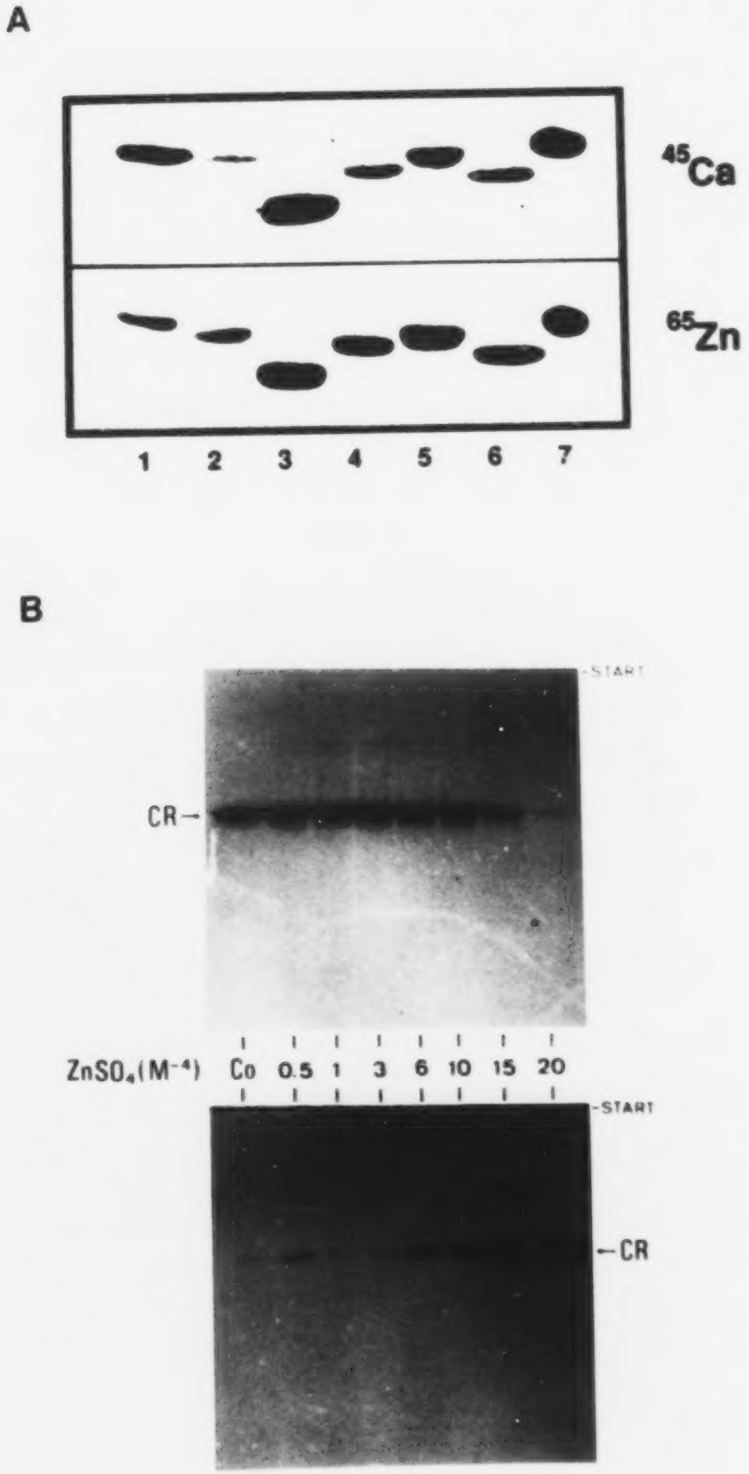
RESULTS

$^{65}Zn^{2+}$ overlay analysis of various calreticulin species - Purified pig pancreatic, bovine, rat and human liver calreticulins were tested for $^{65}Zn^{2+}$ binding by the overlay technique. Figure 5-1A shows $^{45}Ca^{2+}$ (upper panel) and $^{65}Zn^{2+}$ binding to the purified calreticulins as compared to purified calsequestrin, a Ca^{2+} binding protein of the sarcoplasmic reticulum of muscle cells (Jones and Cala, 1981; Milner *et al*, 1991). Purified pig, human, bovine and rat liver calreticulins (Fig. 5-1A, lanes 2, 4, 5, and 6, respectively) bound $^{65}Zn^{2+}$ and $^{45}Ca^{2+}$ under the overlay conditions. Skeletal muscle calsequestrin (Fig. 5-1A, lane 1) and cardiac calsequestrin (Fig. 5-1A, lane 3) bound $^{65}Zn^{2+}$ and $^{45}Ca^{2+}$ under these conditions. $^{65}Zn^{2+}$ and $^{45}Ca^{2+}$ were also carried out with purified skeletal muscle SR proteins and showed calsequestrin as a major $^{65}Zn^{2+}$ and $^{45}Ca^{2+}$ binding protein in this membrane preparation (Fig. 5-1A, lane 7).

Figure 5-1: $^{65}Zn^{2+}$ to Calreticulin and Calsequestrin and Zn^{2+}-Dependent Precipitation of Calreticulin

(A) Proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and incubated with $^{45}Ca^{2+}$(upper panel) or 100 μM $^{65}Zn^{2+}$ (lower panel). Lane 1, purified skeletal muscle calsequestrin; lane 2, pig pancreatic calreticulin; lane 3, canine cardiac calsequestrin; lane 4, human liver calreticulin; lane 5, bovine liver calreticulin; lane 6, rat liver calreticulin; lane 7, rat skeletal muscle sarcoplasmic vesicles.

(B) Purified calreticulin was incubated in the presence of increasing concentration of Zn^{2+} followed by centrifugation. Calreticulin was identified in the supernatants (upper panel) and the pellets (lower panel) by blue staining with Stains-All. Co, control; CR, calreticulin.


A
45Ca
65Zn
1
2
3
4
5
6
7
B
START
CR
ZnSO4(M-4)
Co
0.5
1
3
6
10
15
20
START
CR

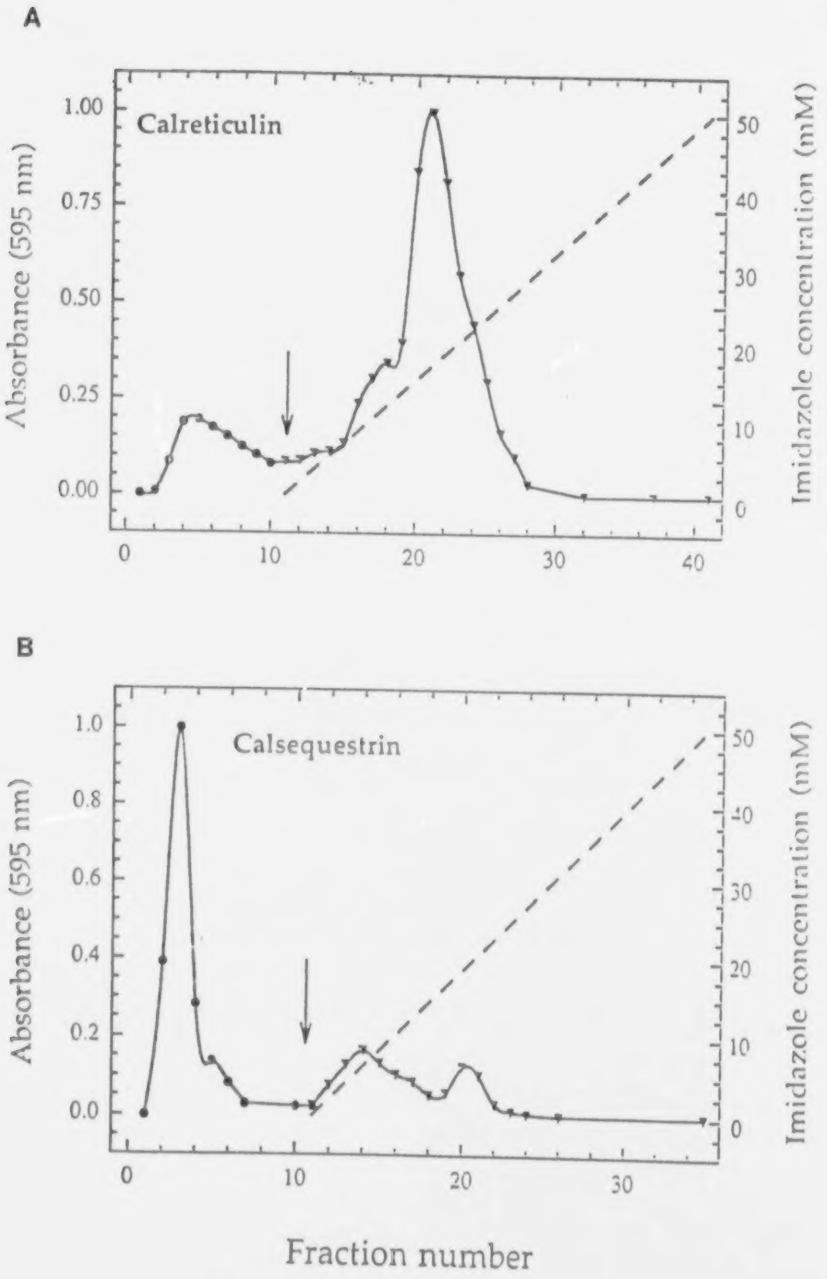
Zn^{2+}-Dependent Precipitation of Calreticulin - Calreticulin undergoes a Ca^{2+}-dependent aggregation and precipitates (Heilmann *et al.*, 1993). In this study, we tested effects of Zn^{2+} on aggregation of calreticulin. Purified protein was incubated in the presence of different concentrations of Zn^{2+} followed by centrifugation and SDS-PAGE of the supernatants and pellets. Calreticulin was identified by its characteristic blue staining with Stains-All (Krause *et al.*, 1990; Milner *et al.*, 1991). Figure 5-1B shows that calreticulin precipitated at approximately 600 μM concentration of Zn^{2+}. In contrast, calsequestrin precipitated at approximately 200 μM Zn^{2+} (Baksh *et al.*, 1995). Calreticulin was precipitated quantitatively at ≥ 2 mM concentration of Zn^{2+} (Fig. 5-1B).

Zn^{2+} Chelating Chromatography of Calreticulin - Interactions of recombinant calreticulin with Zn^{2+} was investigated by Zn^{2+} chelating chromatography in IDA-agarose (Porath and Olin, 1983; Hemdan *et al.*, 1989; Picello *et al.*, 1992). Zn^{2+}-IDA-agarose has been useful in the study of Zn^{2+} binding proteins (Porath and Olin, 1983; Porath *et al*, 1975). In Fig. 5-2A, recombinant calreticulin bound to the Zn^{2+}-IDA-agarose and was eluted with an increasing gradient of imidazole concentration. Calreticulin eluted at approximately 22 mM imidazole (Fig. 5-2A) confirming that the protein interacted with Zn^{2+} under native conditions. Calreticulin's homologue within skeletal muscle, calsequestrin, failed to bind to the Zn^{2+}-IDA-agarose column and comes out in the flowthrough (Fig. 5-2B). Even though calsequestrin has been shown to bind a large amount of Zn^{2+} (Ikemoto *et al.*, 1974;

Figure 5-2: Zn^{2+}-Dependent Chromatography of Calreticulin and Calsequestrin

(A) Recombinant calreticulin was loaded onto a Zn^{2+}-IDA agarose column, equilibrated with 50 mM NaH_2PO_4, pH 7.0 and 100 mM NaCl (fractions 1 to 11) followed by elution with a linear (0 - 50 mM) imidazole gradient (arrow). One-milliliter fractions were collected and assayed for protein content (Bradford, 1976) which was monitored at 595 nm.

(B) Calsequestrin was loaded onto a Zn^{2+}-IDA agarose column, equilibrated with 50 mM NaH_2PO_4, pH 7.0 and 100 mM NaCl (fractions 1 to 11) followed by elution with a linear (0 - 50 mM) imidazole gradient (arrow). One-milliliter fractions were collected and assayed for protein content (Bradford, 1976) which was monitored at 595 nm.


A
Calreticulin
Absorbance (595 nm)
1.00
0.75
0.50
0.25
0.00
Imidazole concentration (mM)
50
40
30
20
10
0
0
10
20
30
40
B
Calsequestrin
Absorbance (595 nm)
1.0
0.8
0.6
0.4
0.2
0.0
Imidazole concentration (mM)
50
40
30
20
10
0
0
10
20
30
Fraction number

and Baksh *et al.*, 1995), it appeared that the two proteins might interact differently with the Zn^{2+}-IDA-agarose. They must therefore coordinate Zn^{2+} in different ways.

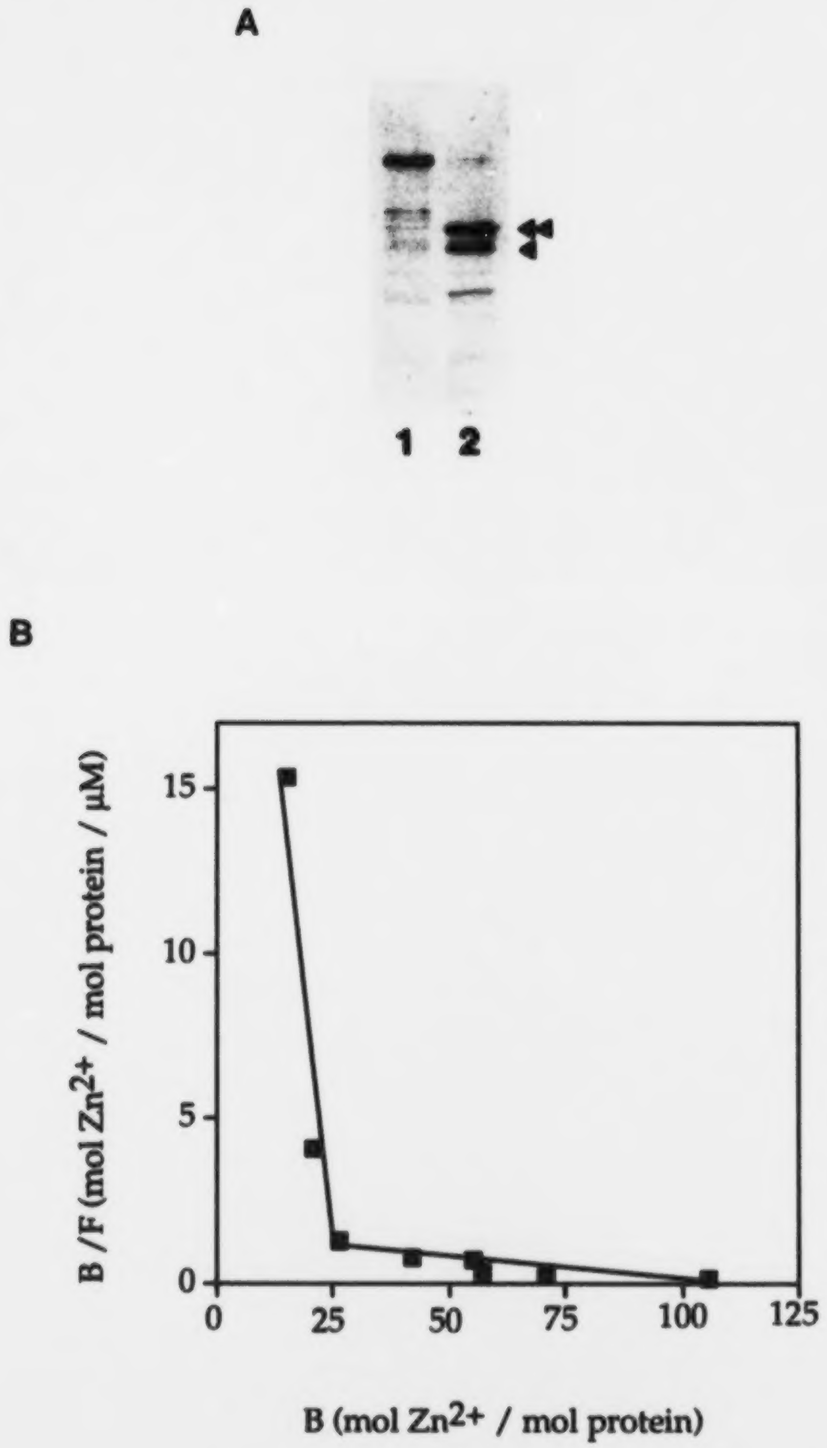
Zn^{2+} Binding to the N-domain of Calreticulin - Zn^{2+} binding to the N-domain was investigated by $^{65}Zn^{2+}$ overlay. The GST-N-domain of calreticulin was purified by Glutathione-Sepharose affinity chromatography and then proteolytically cleaved with Factor Xa to remove GST (see "Chapter Two: Experimental Procedures" for details). Figure 5-3A shows that the GST-N-domain (Fig 5-3A, lane 1) and the isolated the N-domain (Fig. 5-3A, lane 2, single arrow) bound $^{65}Zn^{2+}$ by the overlay technique. GST also bound Zn^{2+} under the conditions used and is shown in lane 2 (double arrow). Under different buffer conditions (Gong and Hew, 1994) and using the SACH assay, GST was shown not to bind Zn^{2+}.

Zn^{2+} Binding to Recombinant and Canine Pancreatic Calreticulin - In order to analyze kinetics of Zn^{2+} binding to calreticulin we have synthesized a Zn^{2+}-sensitive fluorescence dye SACH. This dye was originally developed by Gonzalez *et al.* (1984) to measure free Zn^{2+} concentration in a solution. To our knowledge the use of SACH for measurements of Zn^{2+} levels between 30 - 1000 pg/ml in solution is the most sensitive method currently available (de Torres *et al.*, 1990). The recombinant and canine pancreatic calreticulin used in this study was of high purity and did not contain any detectable protein contamination as determined by SDS-PAGE, its NH_2-terminal amino acid sequence

Figure 5-3: ${}^{65}Zn^{2+}$ binding to the N-domain of calreticulin and Zn^{2+} binding to calreticulin

(A) The N-domain of calreticulin was expressed in E. coli, purified and digested with factor Xa as described under "Experimental Procedures". Proteins were separated by SDS-PAGE (12.5 % gel), transferred to nitrocellulose membranes and incubated with 100 μM ${}^{65}Zn^{2+}$. Lane 1, purified GST-N-domain fusion protein; lane 2, GST-N-domain fusion protein digested with factor Xa (16 hours). Double arrow head, GST. Single arrow head, N-domain.

(B) Zn^{2+} binding to recombinant calreticulin (42 nM) was carried out using the Zn^{2+} sensitive dye SACH as described under "Experimental Procedures." Scatchard analysis of the Zn^{2+} binding data is presented. B, bound; B/F, bound/free. The saturation curve had a biphasic characteristic indicative of the presence of two classes of binding sites.


A
1
2
B
B /F (mol Zn^{2+} / mol protein / µM)
15
10
5
0
0
25
50
75
100
125
B (mol Zn^{2+} / mol protein)

analysis, its reverse phase HPLC chromatography profiles, its reactivity with anti-calreticulin antibodies, and its Ca^{2+} binding properties (Baksh *et al.*, 1992). Using a range of Zn^{2+} concentrations we found that recombinant calreticulin bound 26 moles of Zn^{2+} / mole of protein with high affinity (Kd = 0.8 μM) (Fig. 5-3B). A relatively low affinity (Kd = 47.6 μM) and high capacity (83 moles of Zn^{2+}/mole of protein) Zn^{2+} binding site was also found in the recombinant protein (Fig. 5-3B). The tissue isolated protein, however, revealed only a single class of Zn^{2+} binding sites - binding 23.2 mols of Zn^{2+}/mole of protein with a K_d of 66 μM. Zn^{2+} binding to tissue calreticulin was in agreement with earlier observations (Khanna *et al.*, 1986). However, these authors described only one Zn^{2+} binding site in calreticulin (K_d = 300 μM; B_{max} = 14 moles of Zn^{2+} / mole of protein). The reason for these discrepancies is not clear but may be due to different methods used for measurement of Zn^{2+} binding. Khanna *et al.* (1986) used $^{65}Zn^{2+}$ equilibrium dialysis methods versus the Zn^{2+}-sensitive fluorescence dye used in this study. Furthermore, we have used a relatively low concentration of calreticulin (1 mg / ml) in the assay buffer in order to avoid any Zn^{2+}-dependent precipitation of the protein. Discrepancies in the values reported by Khanna *et al.* (1986) and us (this study) for the Zn^{2+} binding to calreticulin are unlikely due to the use of the Zn^{2+}-sensitive fluorescence dye SACH. We, however, could not explain the absence of the low affinity site within the tissue isolated protein. Specificity of SACH for determination of Zn^{2+} binding to proteins was illustrated using bovine brain S100a, a Ca^{2+} and Zn^{2+} binding protein (Mani and Kay, 1983; Baudier and Gerard, 1986). Analysis of Zn^{2+} binding to S100a using the dye revealed that S100a bound 3.3 moles of Zn^{2+} / mole of protein with a K_d ~ 29 µM. This is in a good agreement with earlier reports (Baudier and Gerard, 1986). We concluded, therefore, that calreticulin has two distinct classes of Zn^{2+} binding sites.

Zn^{2+} Binding to the N+P- and P+C-Domains of Calreticulins - To further distinguish the localization of the two classes of Zn^{2+} binding sites in calreticulin we have employed fluorescence dye SACH to measure Zn^{2+} binding to the N+P- and P+C-domains of calreticulin (free of GST). The N+P-domain bound up to 27 moles of Zn^{2+} / mole of protein (Fig. 5-4A). Scatchard analysis of the binding data revealed that the N+P-domain bound Zn^{2+} with a K_d= ~7.5 µM (Fig. 5-4A, inset). Figure 5-4B shows Zn^{2+} binding to the P+C-domain. This domain bound Zn^{2+} with high capacity (105 moles of Zn^{2+} / mole of protein) and an intermediate affinity (K_d= ~16 µM) (Fig. 5-4B). Since the P-domain, and GST did not bind any Zn^{2+} under these conditions we concluded that the N- and C-domains are responsible for Zn^{2+} binding to calreticulin (see Table 5-1 for a summary of binding constants).

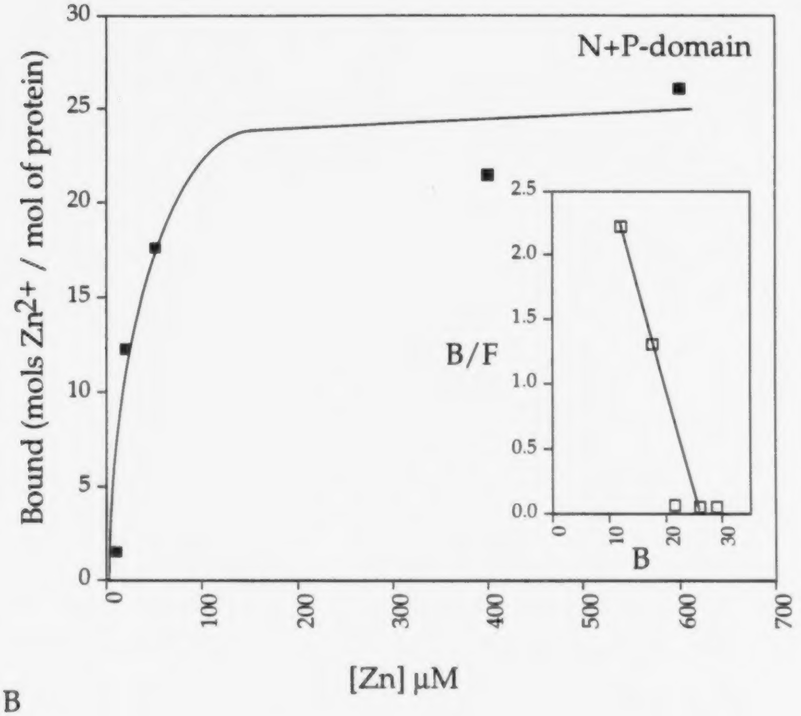
Zn^{2+}-IDA-Agarose Chromatography of Distinct Domains of Calreticulin - In order to identify specific domains in calreticulin that might be responsible for Zn^{2+} binding we utilized a Zn^{2+}-IDA-agarose chromatography and fusion protein approaches. Three regions of calreticulin were expressed as GST fusion proteins and purified: the GST-N+P-domain (amino acid residues 1 to 290), GST-P-domain (amino acid residues 180-273), GST-C-domain (amino acid residues 290 to 401) (Baksh

Figure 5-4: Zn^{2+} Binding to the N+P-Domain and to the P+C-Domain of Calreticulin

(A) Zn^{2+} binding to the purified N+P-domain (63 nM) of calreticulin was carried out using the fluorescence dye SACH as described under "Experimental Procedures". Inset - Scatchard analysis of Zn^{2+} binding data. B, bound; B/F, bound/free.

(B) Zn^{2+} binding to the purified N+P-domain (47 nM) of calreticulin was carried out using the fluorescence dye SACH as described under "Experimental Procedures". Inset - Scatchard analysis of Zn^{2+} binding data. B, bound; B/F, bound/free.

A


N+P-domain
Bound (mols Zn2+ / mol of protein)
0
5
10
15
20
25
30
0
100
200
300
400
500
600
700
[Zn] μM
B/F
0.0
0.5
1.0
1.5
2.0
2.5
0
10
20
30
B

B

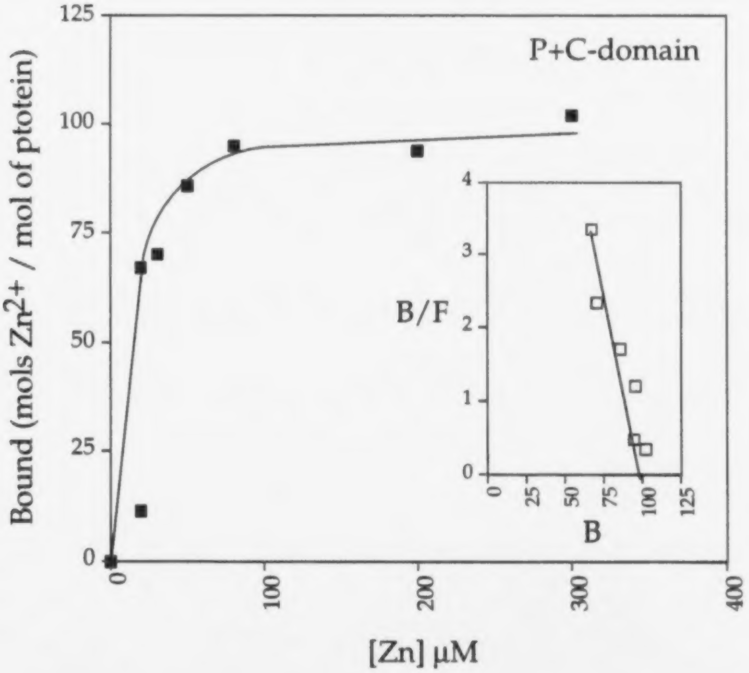

P+C-domain
Bound (mols Zn2+ / mol of ptotein)
0
25
50
75
100
125
0
100
200
300
400
[Zn] μM
B/F
0
1
2
3
4
0
25
50
75
100
125
B

Table 5-1:

Zn^{2+} binding to calreticulin and calreticulin domains

Protein	High affinity site		High capacity site	
	K_d μM	B_{max} mol Zn^{2+}/mol of protein	K_d μM	B_{max} mol Zn^{2+}/mol of protein
Recombinant calreticulin	0.8	26	47	83
Tissue calreticulin	66	23.2	---	---
N+P-domain	7	27	---	---
P+C-domain	---	---	16	105

Note: All these samples were cleaved with Factor Xa, and GST removed by application on a Glutathione S-transferase affinity column. Recombinant calreticulin domains were further purified on a Mono Q FPLC column (see "Chapter Three: Purification, expression, and biochemical characterization of calreticulin and calreticulin domain). "---"denotes no Zn^{2+} binding to the protein.

and Michalak, 1991). Prior to Zn^{2+}-IDA-agarose chromatography the fusion proteins were purified on the Glutathione-Sepharose affinity column followed by digestion with Factor Xa as described in "Chapter Two: Experimental Procedures".

N+P-domain - Figure 5-5A shows interaction of the N+P-domain with the Zn^{2+}-IDA-agarose. Under the conditions used in this study digestion of the N+P-domain with factor Xa resulted in appearance of three major protein bands of molecular weight of 36 000, 31 000 and 26 000 (Fig. 5-5A, lanes 2 and 3). A 26-kDa protein band corresponded to the recombinant GST. The identity of the 36- and 31-kDa protein bands was established by NH_2-terminal amino acid sequence analysis. The NH_2-terminal amino acid sequence of the 39-kDa protein band was E-P-A-I-Y-K. This sequence was identical to the NH_2-terminal amino acid sequence of calreticulin (Michalak *et al.*, 1992) and we have identified this protein band as the full length N+P-domain (Fig. 5-5A, double arrow head). The NH_2-terminal amino acid sequence of the 31-kDa protein band was F-Y-A-L-S-A-. This sequence corresponded to amino acid sequence from residue 57 to residue 62 within mature calreticulin (Michalak *et al.*, 1992) and was referred to as the truncated N+P-domain (Fig. 5-5A, arrow head). It was not clear why factor Xa digestion of the N+P-domain produced the truncated N+P-domain. However, prolonged cleavage times produced 100% of the truncated N+P-domain and this may be due to a factor Xa nonspecific cleavage site within the full length N+P-domain fusion protein.

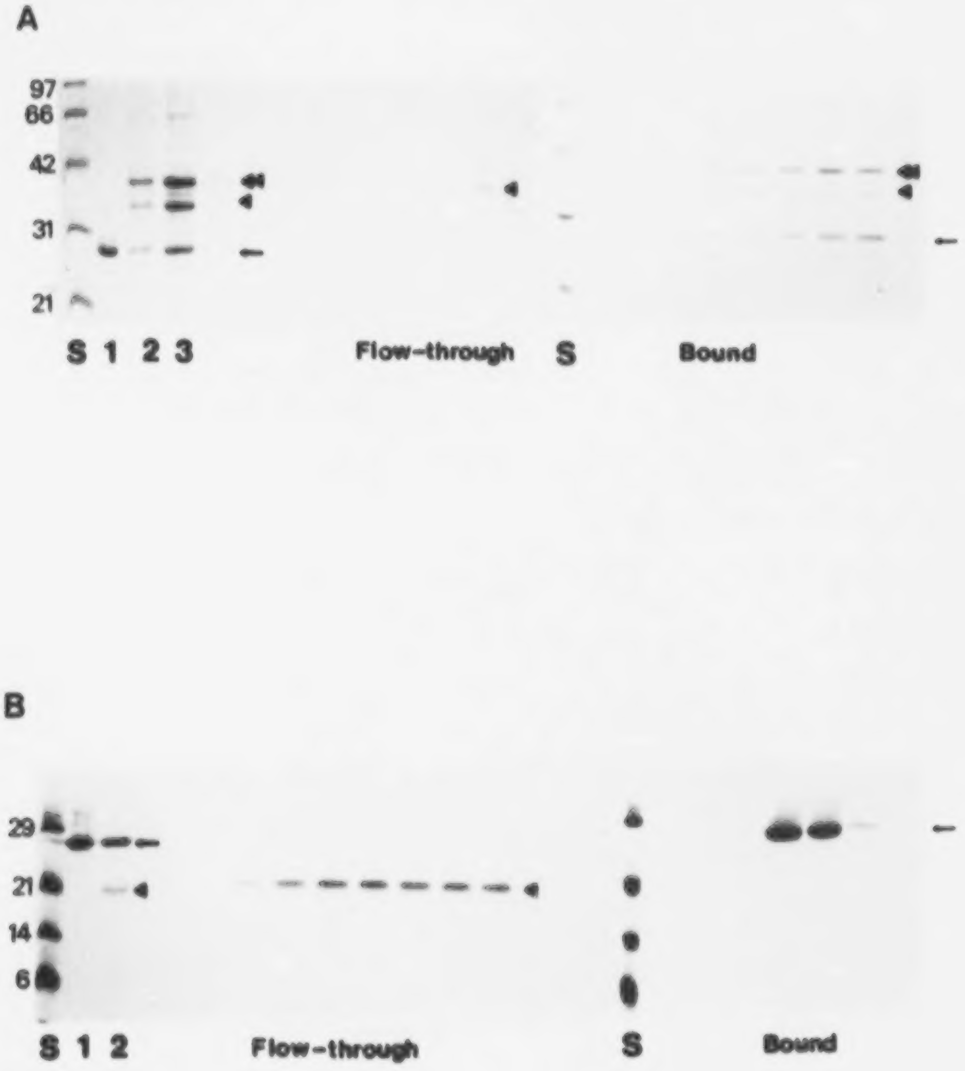
To identify if the N+P-domain bound Zn^{2+}, a mixture of the N+P-domain, truncated N+P-domain and GST (Fig. 5-5A, lanes 2 and 3) was separated by Zn^{2+}-IDA-agarose chromatography. Zn^{2+} binding proteins interact with Zn^{2+}-IDA-agarose and are eluted from the column with an increasing concentration of imidazole (Porath and Olin, 1983; Hemdan *et al.*, 1989; Picello *et al.*, 1992). The "flow-through" fractions from Zn^{2+}-IDA-agarose contained proteins that either did not bind Zn^{2+} or interacted very weakly with Zn^{2+}. All three proteins (N+P-domain, truncated N+P-domain and GST) bound to the Zn^{2+}-IDA-agarose and were eluted with imidazole (Fig. 5-5A, Bound fractions). It was shown in Fig. 5-3A that GST may bind Zn^{2+} as determined using the $^{65}Zn^{2+}$ overlay technique and hence it is not surprising that it did appear in the elution. The full length N+P-domain and the GST were the major proteins found in the "Bound" fractions. The major protein found in the "flow-through" fractions (~70%) was the truncated N+P-domain suggesting that this domain (missing the NH_2-terminal 56 amino acid residues) interacted only weakly with the Zn^{2+}-IDA-agarose (Fig. 5-5A, "Flow-through" fractions, arrow head).

P-domain - In order to establish a role of the P-domain in Zn^{2+} binding to calreticulin the GST-P-domain fusion protein was digested with factor Xa, purified by the Glutathione-Sepharose affinity chromatography and subjected to Zn^{2+}-IDA-agarose chromatography. Figure 5-5B showed that the P-domain of calreticulin did not bind to the Zn^{2+}-IDA-agarose and was found in the "flow-through" fractions (Fig. 5-5B, arrow head). The identity of the P-domain protein band in the "flow-through" fractions was confirmed by the NH_2-terminal amino acid sequence analysis. As expected the 26-kDa GST was found in the bound and eluted fractions (Fig. 5-5B, small arrow).

Figure 5-5: Zn^{2+}-IDA Agarose Chromatography of the N+P-domain and P-domain of Calreticulin

(A) The N+P domain of calreticulin was expressed in *E. coli* and purified by Glutathione-Sepharose affinity chromatography. The protein was subjected to digestion with Factor Xa (10 hours) followed by Zn^{2+}-IDA-agarose chromatography as described under "Experimental Procedures". Unbound material ("Flow-through" fractions), bound and eluted fractions ("Bound" fractions) were separated in SDS-PAGE (12.5 % gel) followed by staining with Coomassie blue. Lane 1, purified recombinant GST; lane 2 and 3, purified, factor Xa digested GST-N+P-domain fusion protein (sample loaded onto the Zn^{2+}-IDA-agarose); S, low molecular Bio-Rad protein standards. Double arrow head, full length N+P-domain. Arrow head, truncated N+P-domain. Arrow indicates the position of GST.

(B) The P domain of calreticulin was expressed in *E. coli*, purified by Glutathione-Sepharose affinity chromatography, followed by digestion with factor Xa (16 hours). The digested protein was used for Zn^{2+}-IDA-agarose chromatography as described under "Experimental Procedures". "Flow-through" fractions (unbound material) and "Bound" fractions (bound and eluted fractions) were analyzed by SDS-PAGE (15 % gel). Lane 1, purified recombinant GST; lane 2, purified, factor Xa digested GST-P-domain fusion protein (sample loaded onto the Zn^{2+}-IDA-agarose); S, Mandel low molecular weight protein standards. Arrow head, P-domain. Arrow indicates the position of GST.


A
97
66
42
31
21
S
1
2
3
Flow-through
S
Bound
B
29
21
14
6
S
1
2
Flow-through
S
Bound

C-domain - Figure 5-6A showed the Zn^{2+}-IDA-agarose chromatography of the factor Xa treated GST-C-domain of calreticulin. Similar to the N+P-domain, the C-domain of calreticulin was found in the bound and eluted fractions (Fig. 5-6A, arrow head) together with the GST (small arrow). Based on the Zn^{2+}-IDA-agarose chromatography experiments we concluded that Zn^{2+} binding sites of calreticulin were localized to the N- and C-domains but not to the P-domain of the protein.

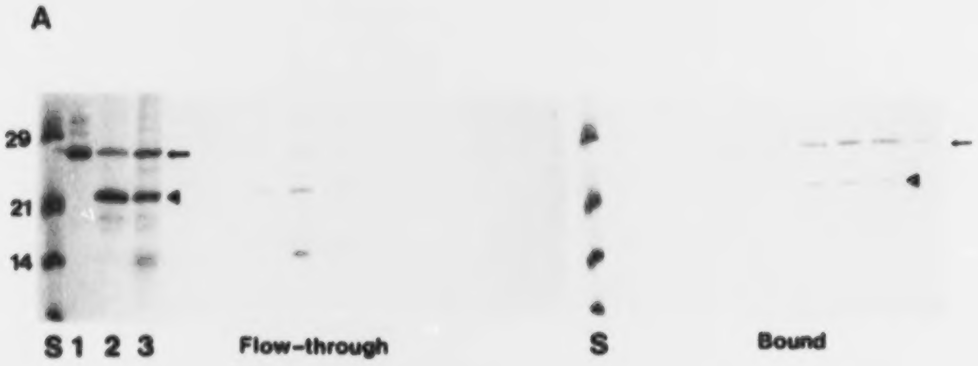
Effect of Ca^{2+} and Mg^{2+} on Zn^{2+} Binding to Calreticulin - Using Zn^{2+} specific fluorescence dye salicylcarbohydrazone (SACH) we have determined that calreticulin had two classes of Zn^{2+} binding sites: the high affinity site (K_d = ~ 0.8 μM; B_{max} = ~ 26 mole Zn^{2+}/mole protein) and the intermediate affinity and high capacity site (K_d = ~ 48 μM; B_{max} = ~ 83 mole Zn^{2+}/mole protein). Figure 5-6B showed the effect of Ca^{2+} and Mg^{2+} on Zn^{2+} binding to calreticulin. Ca^{2+} and Mg^{2+} affected the intermediate affinity/high capacity Zn^{2+} binding to calreticulin (Fig. 5-6B, right panel) but had no effect on the high affinity sites (Fig. 5-6B, left panel). Ca^{2+} and Mg^{2+} reduced Zn^{2+} binding to the high capacity site by approximately 80% and 52%, respectively (Fig. 5-6B). These observations suggested that the low capacity site may not be physiologically significant and that the Zn^{2+} and Ca^{2+} binding sites may be localized to different regions in calreticulin.

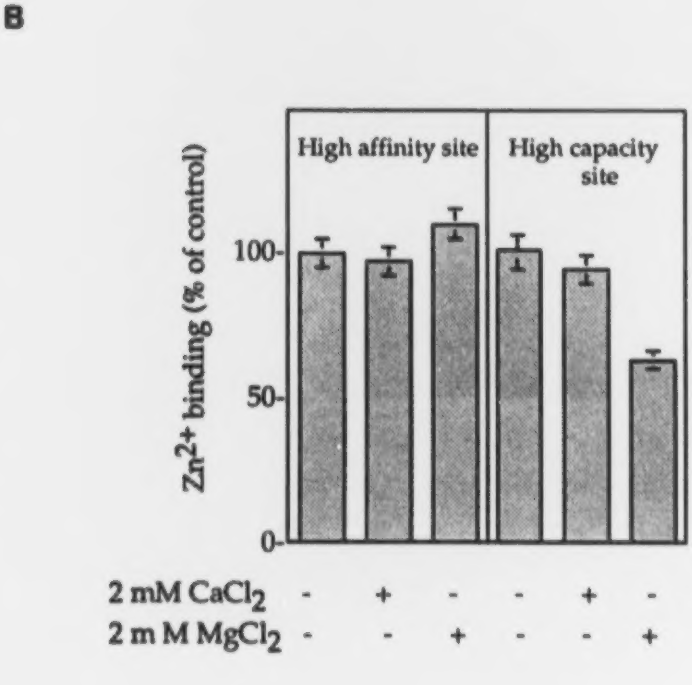
Zn^{2+}-Dependent Conformational Changes in Calreticulin - Khanna *et al.* (1986) reported that calreticulin (calregulin) undergoes a Zn^{2+}-dependent conformational changes as measured by fluorescence

Figure 5-6: Zn^{2+}-IDA Agarose Chromatography of C-domain of calreticulin and effects of Ca^{2+} and Mg^{2+} on Zn^{2+} binding to calreticulin

(A) The GST-C domain fusion protein of calreticulin was expressed in *E. coli*, purified by Glutathione-Sepharose affinity chromatography and digested with factor Xa (16 hours). The digested protein (lane 2 and 3) was used for Zn^{2+}-IDA-agarose chromatography as described under "Experimental Procedures". "Flow-through" fractions (unbound material) and "Bound" fractions (bound and eluted fractions) were analyzed by SDS-PAGE. Lane 1, purified recombinant GST; lane 2 and 3, purified, factor Xa digested GST-C-domain fusion protein (sample loaded onto the Zn^{2+}-IDA-agarose); S, Mandel low molecular weight protein standards as indicated. A small portion of the C-domain and GST found in the "flow-through" fractions is due to overloading of the Zn^{2+}-IDA-agarose column. Arrow head, C-domain. Arrow indicates the position of GST.

(B) Zn^{2+} binding to calreticulin was measured in the absence (-) and presence (+) of 2 mM Ca^{2+} or 2 mM Mg^{2+} using the fluorescence dye SACH as described under "Experimental Procedures". For the representation of the high affinity and high capacity Zn^{2+} binding sites, analysis was carried out in the presence of 1 μM and 50 μM Zn^{2+} concentrations, respectively. 100% Zn^{2+} binding represents Zn^{2+} binding in the absence of competing metals.


A
29
21
14
S 1 2 3
Flow-through
S
Bound


B
High affinity site
High capacity site
Zn2+ binding (% of control)
100
50
0
2 mM CaCl2 - + - - + -
2 m M MgCl2 - - + - - +

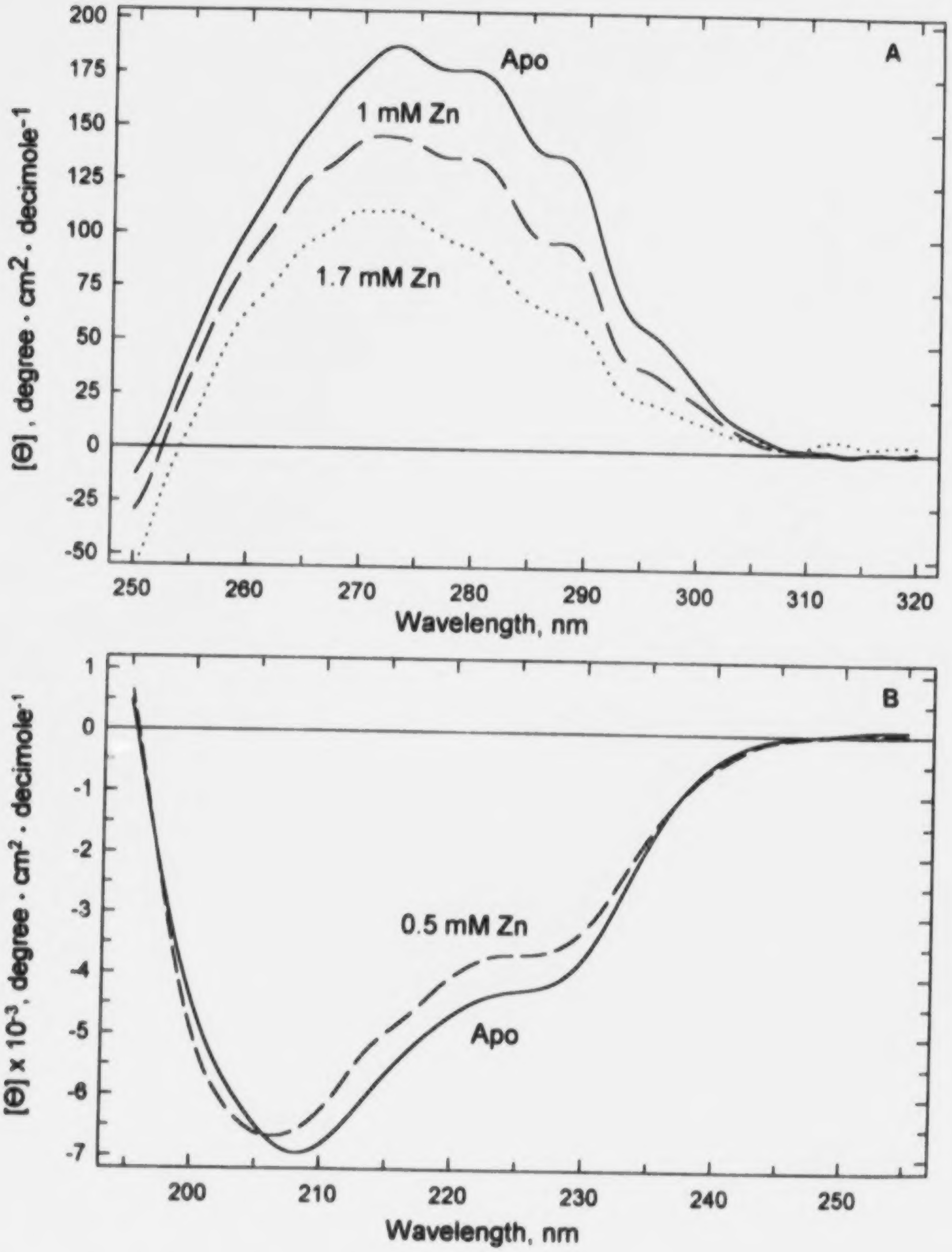
spectroscopy. Analysis of Zn^{2+}-dependent changes in CD spectra of calreticulin revealed minor alterations in elements of secondary structure as reflected by very small far UV CD changes. However, subtle alterations occurred in the environment around key aromatic residues since dramatic quenching of the CD signal occured in this spectral region. As shown in Figure 5-7B the far UV CD spectrum of calreticulin displayed a minimum near 208 nm and a shoulder around 225 nm. Application of the contin analysis gave the following figures for the amount of the various confomers: α-helix 11%, ß-sheet 42 %, ß-turn 26% and 21% remainder. In the presence of 0.5 mM Zn^{2+} the minimum was shifted to ~ 206 nm. There was an overall slight reduction in ellipticity. This translated to 9% α-helix, 43% ß-sheet, 24% ß-turn and 24% remainder.

The aromatic CD spectrum of calreticulin (Fig. 5-7A) was characterized by a broad peak at 277 nm with distinct shoulders at 281 nm, 289 nm and 296 nm. These peaks were due to trp/tyr residues in asymmetric environments in the protein structure. In Fig. 5-7A, addition of Zn^{2+} dramatically quenched the near UV CD signal, although the overall shape of the spectrum was unaltered. These findings further supported that calreticulin is a Zn^{2+}-binding protein.

Zn^{2+}-Dependent Conformational Changes in Calreticulin Domains - Figure 5-8A described the far UV CD spectrum of the N+P-domain of calreticulin (free of GST). It displayed a minimum near 208 nm and a shoulder around 225 nm. This was very similar to the far UV CD spectrum for recombinant calreticulin (Fig. 5-7A). Application of the contin analysis gave the following figures for the amount of the various

Figure 5-7: Zn^{2+}-Dependent Conformational Changes in Calreticulin

CD measurements were carried out as described under "Experimental Procedures". In A, near UV CD spectra of calreticulin in the absence (—) and presence of 1 mM Zn^{2+} (----) and 1.7 mM Zn^{2+} (····). In B; far UV CD spectra of calreticulin in the absence (—) and presence of 0.5 mM Zn^{2+} (----).


A
Apo
1 mM Zn
1.7 mM Zn
[Θ] , degree · cm^2 · decimole^{-1}
200
175
150
125
100
75
50
25
0
-25
-50
250
260
270
280
290
300
310
320
Wavelength, nm
B
0.5 mM Zn
Apo
[Θ] x 10^{-3}, degree · cm^2 · decimole^{-1}
1
0
-1
-2
-3
-4
-5
-6
-7
200
210
220
230
240
250
Wavelength, nm

conformers: α-helix 7%, ß-sheet 45 %, ß-turn 22% and 26% remainder. In the presence of 2 mM Zn^{2+} (Fig. 5-8A) the minimum remained, but the overall shape undergoes an overall slight reduction in ellipticity similar to that for recombinant calreticulin (Fig. 5-7A). This translated to 3% α-helix, 49% ß-sheet, 23% ß-turn and 26% remainder.

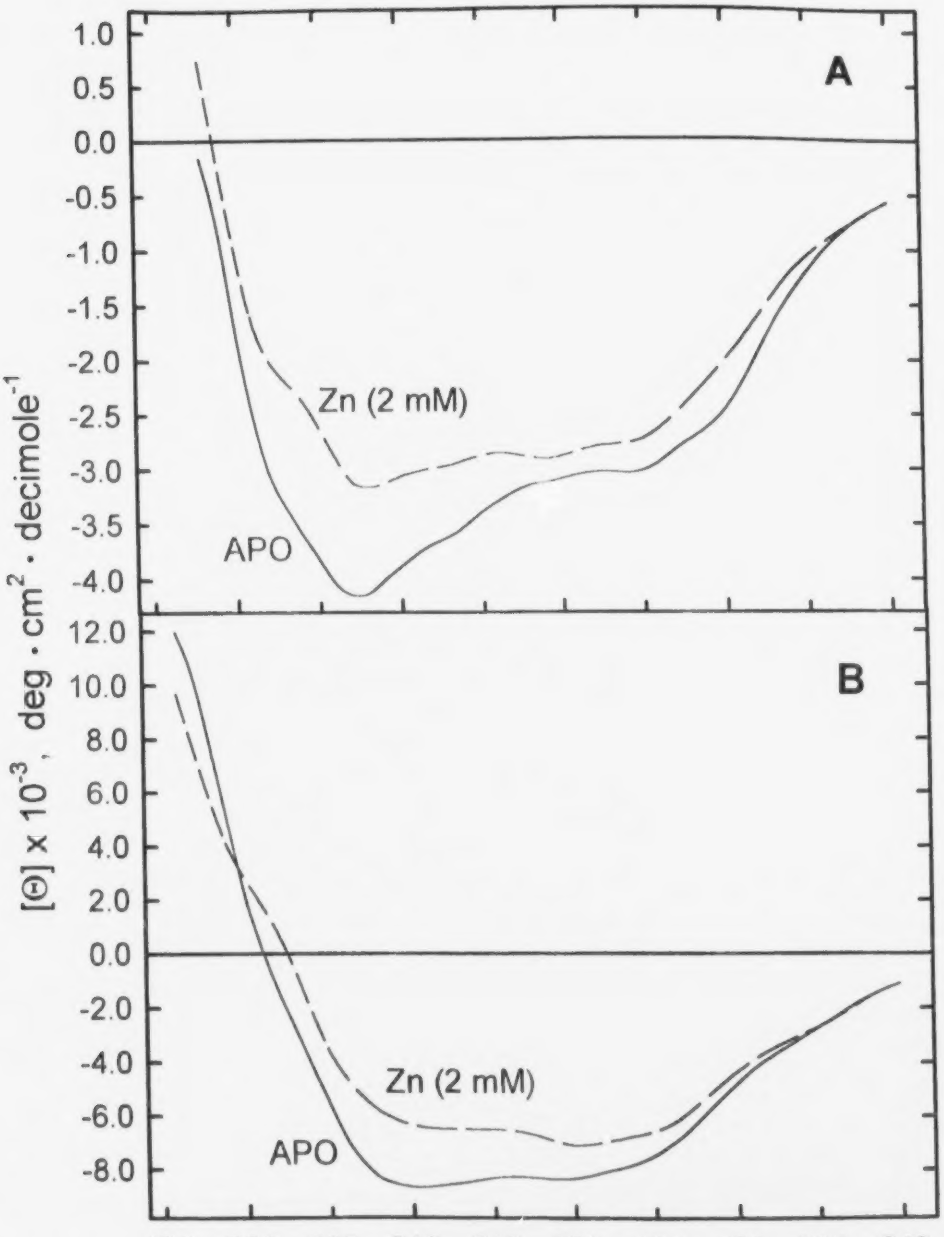
Figure 5-8B described the far UV CD spectrum of the C-domain of calreticulin (free of GST). The presence of a large cluster of acidic residues within this domain of calreticulin resulted in this domain acquiring a helical structure in solution (Fig. 5-8B). This domain did not have a minimum but still had a broad shoulder at 220 nm. Application of the contin analysis gave the following figures for the amount of the various conformers: α-helix 23%, ß-sheet 38 %, ß-turn 10% and 28% remainder. In the presence of 2 mM Zn^{2+} (Fig. 5-8B) the overall shape undergoes an overall slight reduction in ellipticity similar to that for recombinant calreticulin (Fig. 5-7B). This translated to 18% α-helix, 27% ß-sheet, 21% ß-turn and 34% remainder.

It would, therefore, appear that although addition of Zn^{2+} to calreticulin only produced minor alterations in elements of secondary structure as reflected by very small far UV CD changes, subtle alterations do occur in the environment around key aromatic residues since dramatic quenching of the CD signal occurred in this spectral region.

DEPC Modification of Calreticulin - The role of histidines in Zn^{2+} binding to calreticulin was examined by using chemical modification of the protein with DEPC. This method was used by Schiavo *et al.* (1992) to show the importance of histidines in the binding of Zn^{2+} to botulinum

Figure 5-8: Zn^{2+}-Dependent Conformational Changes in Calreticulin Domains

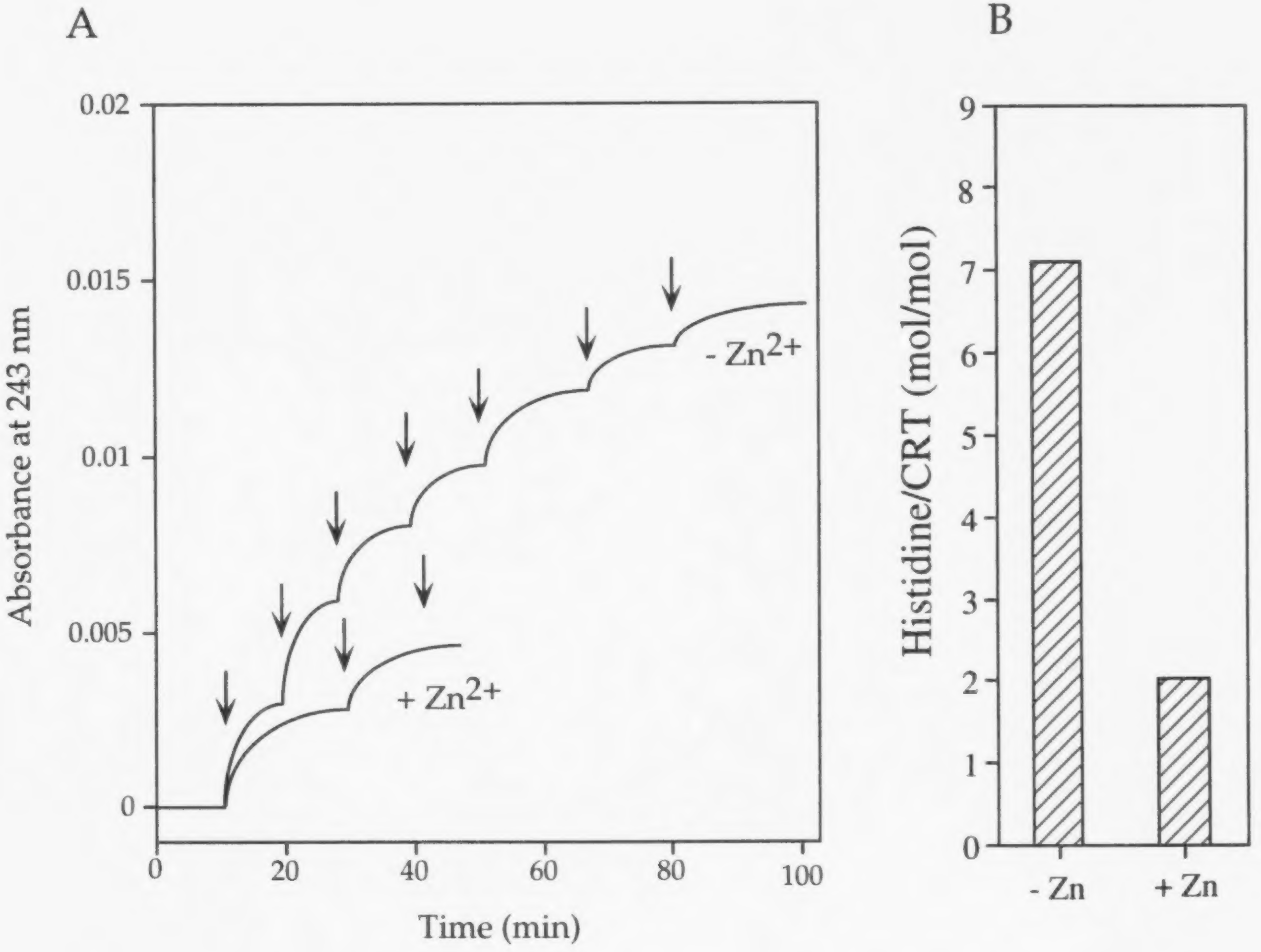
CD measurements were carried out as described under "Experimental Procedures". In A, far UV CD spectra of the N+P-domain of calreticulin in the absence (—) and presence of 2 mM Zn^{2+} (----). In B; far UV CD spectra of the C-domain of calreticulin in the absence (—) and presence of 2 mM Zn^{2+} (---).


A
B
Zn (2 mM)
APO
Zn (2 mM)
APO
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
-2.0
-4.0
-6.0
-8.0
[Θ] x 10⁻³, deg · cm² · decimole⁻¹
195
200
205
210
215
220
225
230
235
240
Wavelength, nm

neurotoxins. It was also used by Shashan-Barmatz and Weil (1994) to show the involvement of histidines in the inactivation of the ryanodine receptor/Ca^{2+} channel from skeletal muscle. DEPC modifies histidine and to a much lesser extent tyrosine residues in protein and coverts them to N-carbethoxyhistidine and O-carbethoxytyrosine, respectively. N-carbethoxyhistidine and O-carbethoxytyrosine formation are monitored by characteristic absorbance peaks at 243 nm and 278 nm, respectively. Figure 5-9A shows the concentration dependence of the modification of tissue calreticulin with DEPC as monitored by the increase of the absorbance at 243 nm. No tyrosine residues were modified as there was no change of absorbance at 278 nm (characteristic of the modified form of tyrosine - O-carbethoxytyrosine). In the absence of Zn^{2+}, histidine residues continued to be modified by DEPC and absorbance readings continued to rise indicating the formation of N-carbethoxyhistidine. In the absence of Zn^{2+}, seven additions of DEPC produced a sequential rise in the absorbance at 243 nm. Further additions produced a sharp drop in the absorbance at 243 nm to basal levels. In the presence of Zn^{2+}, only two levels of addition gave rises in the absorbance readings and the readings then dropped to basal. Based on the absorbance spectra in Fig. 5-9B it was estimated that 7 moles of histidine / mole of calreticulin were modified in the absence of Zn^{2+}, but only two moles of histidines / mole of calreticulin were modified in the presence of Zn^{2+} (Fig. 5-9B, right panel). We concluded that 5 out of 7 histidines were inaccessible and thus protected from DEPC modification in the presence of Zn^{2+} and thus may be involved in Zn^{2+} binding to calreticulin. The remaining two histidines appear to be accessible to DEPC modification when Zn^{2+} is

Figure 5-9: DEPC Modification of Histidines in Calreticulin

DEPC titration of dog pancreatic calreticulin was carried out as described under "Experimental Procedures" In A, the increase in absorbance at 243 nm indicates the progressive formation of N-carbethoxyhistidine. Successive 10 mM additions of DEPC are indicated by arrows. In (B), the absorbance results in (A) were quantified using the molar extinction coefficient for N-carbethoxyhistidine of 3 200 M^{-1} cm^{-1} and plotted as a mole ratio of amount of histidine modified to amount of tissue calreticulin used in the modification.


A
Absorbance at 243 nm
0.02
0.015
0.01
0.005
0
0
20
40
60
80
100
Time (min)
- Zn2+
+ Zn2+
B
Histidine/CRT (mol/mol)
9
8
7
6
5
4
3
2
1
0
- Zn
+ Zn

bound to the protein. Recombinant calreticulin showed a similar protection of 5 moles of histidine/mole of protein in the presence of Zn^{2+}.

The possible role of cysteines in Zn^{2+} binding to calreticulin was tested using a reduced and alkylated form of the protein, as described in "Chapter Two: Experimental Procedures". Calreticulin has three cysteines two of which may form an intramolecular disulfide bridge (Matsuoka *et al.*, 1994). Zn^{2+} binding to the high affinity/low capacity site (found in the N-domain) was significantly reduced in the reduced and alkylated protein, while similar B_{max} values were obtained for the intermediate affinity/high capacity site. This suggested that cysteine residues may play some indirect structural support role in Zn^{2+} binding to the N-domain of calreticulin.

DISCUSSION

In this study we identified regions in the protein responsible for Zn^{2+} binding (Fig. 5-10). Recombinant calreticulin had two classes of Zn^{2+} binding sites: a relatively high affinity site (K_d = 0.8 µM; B_{max} = 26 moles of Zn^{2+} /mole of protein) and a low affinity site (K_d = 47.6 µM; B_{max} = 83 moles of Zn^{2+}/mole of protein); whereas the tissue isolated protein has one class of binding sites - binding 23.2 mols of Zn^{2+} /mole of protein with a K_d of 66 µM. Mg^{2+} was found to occupy 50% of the high capacity Zn^{2+} site in the presence of Zn^{2+} (Fig. 5-10). Zn^{2+} binding to calreticulin was further confirmed by its interaction with the Zn^{2+}-IDA-agarose and by Zn^{2+}-dependent aggregation (precipitation) of the protein. The ability of Zn^{2+} to precipitate calreticulin is analogous to previously

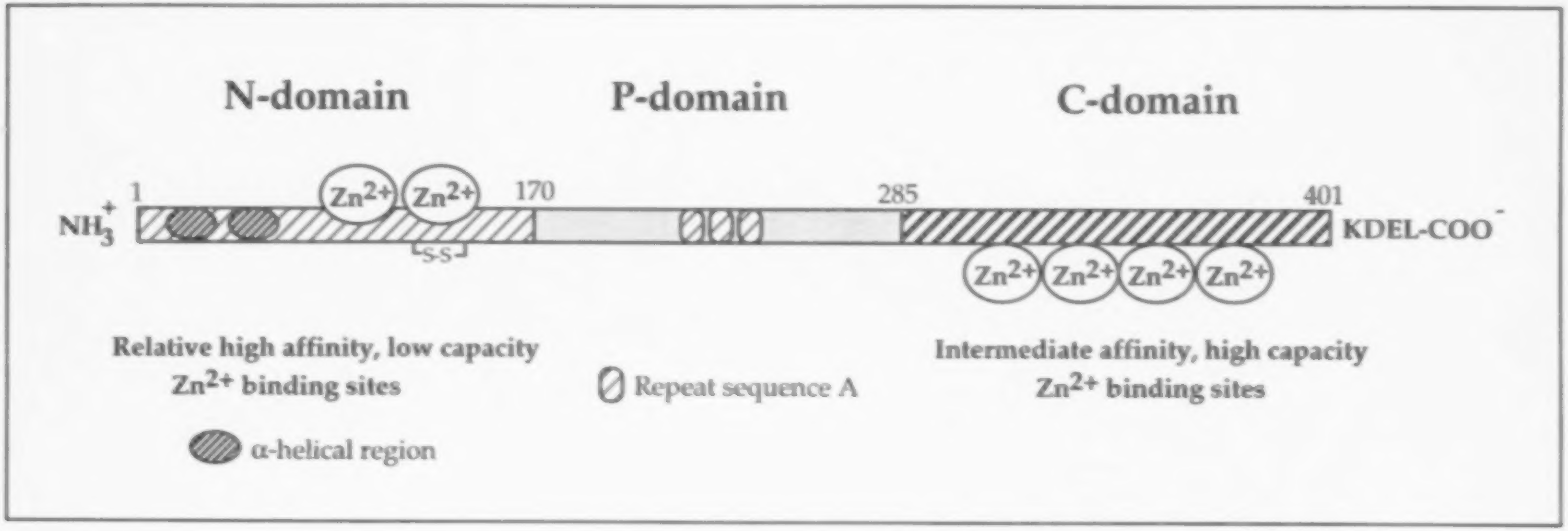

N-domain
P-domain
C-domain
1
170
285
401
NH$_3^+$
KDEL-COO$^-$
Zn^{2+}
Zn^{2+}
S-S
Zn^{2+}
Zn^{2+}
Zn^{2+}
Zn^{2+}
Relative high affinity, low capacity Zn^{2+} binding sites
Repeat sequence A
Intermediate affinity, high capacity Zn^{2+} binding sites
α-helical region

Figure 5-10: Calreticulin domains involved in Zn^{2+} binding

Putative location of the high affinity, low capacity Zn^{2+} binding sites (N-domain) and of the intermediate affinity, high capacity Zn^{2+} binding sites (C-domain) in relation to the Mg^{2+} binding sites in calreticulin are indicated. Numbers indicate amino acid position within rabbit skeletal muscle calreticulin sequence.

reported Ca^{2+}-dependent precipitation of the protein (Heilmann *et al.*, 1993). Khanna *et al.* (1986) showed by fluorescence spectroscopy that calreticulin undergoes Zn^{2+}-dependent conformational changes. We also observed that upon binding of Zn^{2+}, calreticulin undergoes a 3 nm blue shift in the far UV CD spectrum and quenching of the aromatic ellipticity (Fig. 5-7A). This type of change is indicative of a movement of aromatics, particularly the tryptophans and tyrosines to a position away from the solvent. Hence, the structure of the protein becomes more compact upon binding of Zn^{2+}. This property of calreticulin was utilized for a Zn^{2+}-dependent purification of the protein by Phenyl-Sepharose chromatography (Heilmann *et al.*, 1993). Zn^{2+} binding to calreticulin may, therefore, play an important role inducing the folding of the protein or its domains. This is similar to the essential role of Zn^{2+} in the folding of the DNA binding domains of transcription factors and the large family of hormone receptor proteins (Berg, 1990; Coleman, 1992).

Using the GST-fusion protein approach together with Zn^{2+}-IDA-agarose chromatography, followed by analysis of Zn^{2+} binding with the fluorescence dye SACH, we have localized the high affinity Zn^{2+} binding site to the NH_2-terminal region of calreticulin (Fig. 5-5A, and 5-5B). The high capacity Zn^{2+} binding sites were found in the carboxyl-terminal region of the protein (Table 5-1, C-domain). A central P-domain of calreticulin did not bind any Zn^{2+} and did not interact with Zn^{2+}-IDA-agarose indicating that this domain was not involved in Zn^{2+} binding to the protein (Fig. 5-5B). We have also shown that Zn^{2+} binding to the intermediate affinity, high capacity site is the only class of Zn^{2+} binding sites within calreticulin that is reduced in the presence of Ca^{2+} and/or

Mg^{2+} suggesting that this site may not be physiological. It is not clear why the B_{max} values associated with Zn^{2+} binding to calreticulin are high, since the protein sequence does not support the binding of > 100 moles of Zn^{2+}. Calreticulin's homologue in striated muscle cells, calsequestrin, has been found to bind > 150 moles Zn^{2+} / mole of protein (Ikemoto, 1974). Both calsequestrin and S100a were used as controls in the determination of Zn^{2+} binding using the dye SACH. We obtained a B_{max} value of 200 moles of Zn^{2+} / mole of calsequestrin and 3.3 mole Zn^{2+} / mole of S100a protein ($K_d \sim 29\ \mu M$). This was comparable to the values obtained by Ikemoto (1974), for calsequestrin, and Baudier and Gerard (1986), for S100a. It appears that calreticulin may bind a large amount of Zn^{2+} as described in this chapter.

Calreticulin does not have any consensus amino acid sequences such as "Zn^{2+}-fingers" known to be associated with Zn^{2+} binding sites in proteins (Berg, 1990; Coleman, 1992) making it difficult to predict precisely localization of Zn^{2+} binding site(s) in the protein. Results of this study show that an intermediate affinity/high capacity Zn^{2+} binding to calreticulin is restricted to the C-domain. This region of calreticulin also binds Ca^{2+} with a low affinity and high capacity (Baksh and Michalak, 1991). Zn^{2+} binding to the C-domain is documented by the following three observations: first, the C-domain binds to the Zn^{2+}-IDA-agarose; second, the purified P+C-domain binds ~105 moles of Zn^{2+} per mole of protein as measured using fluorescence dye SACH (the P-domain does not bind any Zn^{2+}), and finally, Zn^{2+} binding to the high capacity site is reduced in the presence of Ca^{2+} and Mg^{2+}, ions that bind to the C-domain of the protein (Baksh and Michalak, 1991). The C-domain of

Figure 5-11: Amino acid sequences of potential Zn^{2+} binding regions within calreticulin.

Location of potential histidine residues which may be involved in Zn^{2+} binding to calreticulin is shown in bold in the N-domain. Boxed areas within the N-domain represent areas conserved throughout all calreticulin amino acid sequences obtained so far (Fig. 1-4). The location of acidic residues is shown in bold in the C-domain.

N-domain

10 20 30 40 50 60 70 80
EPVVYFKEQF LDGDGWTERW IESKHKSDFG KFVLSSGKFY GDQEKDKGLQ TSQDARFYAL SARFEPFSNK GQPLVVQFTV

90 100 110 120 130 140 150 160
KHEQNIDCGG GYVKLFPAGL DQKDMHGDSE YNIMFGPDIC GPGTKKVHVI FNYKGKNVLI NKDIRCKDDE FTHLYTLIVR
S-S

170 180
PDNTYEVKID NSQVESGSLE

C-domain

* 330 340 350 360 370 380 390 401
YA**EE**FGN**E**TW GVTKTA**E**KQM KDKQ**DEE**QRL K**EEEEE**KKRK **EEEEAEEDEE** **D**K**DD**K**EDEDE** **DEED**K**DEEEE** **E**AAAGQAK**DE**L

calreticulin does not contain any cysteine and/or histidine residues known to be associated with Zn^{2+} binding sites in a number of different proteins (Berg, 1990; Coleman, 1992). The C-domain, however, contains a large number of negatively charged residues (Fig. 5-11). In the last 57 residues, 37 are aspartic or glutamic acid (Michalak *et al.*, 1992). It is likely that Zn^{2+} binds to the repetitive clusters of glutamic and aspartic acid residues found in this region of calreticulin. Zn^{2+} exhibits very specific preference for ligand moieties in proteins: the thiolate sulfur of cysteines, the imidazole nitrogen atoms of histidines, and the carboxylate oxygens of glutamic and aspartic acid residues. Zn^{2+} involved in the catalytic functions appear to utilize the carboxylate oxygens of glutamic and aspartic acid (Vallee and Auld, 1990a, b). It has been observed in our laboratory that calreticulin undergoes a Zn^{2+} dependent degradation process that is not affected by the presence of serine and cysteine protease inhibitors (Andrin and Michalak, unpublished results). It is possible that Zn^{2+} may, therefore, confer this function to the C-domain of calreticulin. Similar clusters of negatively charged residues are involved in Zn^{2+} binding to a 19-kDa Zn^{2+} binding protein identified in rat liver (Brand *et al.*, 1988) as an inactivator of 6-phosphofructo-1-kinase in a Zn^{2+}-dependent manner. It is possible that in calreticulin Zn^{2+} could be binding to these sites. Zn^{2+} binding to the C-domain may follow cooperative binding since the curve for the P+C-domain may follow a sigmoidal shape (Fig. 6-4B). The B_{max} value for the high capacity site (~105 moles of Zn^{2+}/mole of protein) would thus appear higher than that obtained for the high capacity site for recombinant calreticulin (~ 83 moles of Zn^{2+}/mole protein) if Zn^{2+} binding to the C-domain follows a cooperative nature (Fig. 5-4B and Table 5-1). It is not clear at present why the P+C-domain of calreticulin bound over 20 moles of Zn^{2+} more than the native protein. Different folding conformations of the C-domain within the P+C-domain fusion protein may be activated when it is expressed alone or as a part of the whole native protein. Proper tertiary structure would impose restrictions on "false" cooperative binding when the P+C-domain is linked to the N-domain. Since the P+C-domain of calreticulin is missing the highly structured N-domain it is possible that 105 moles of Zn^{2+} / moles of protein is a "false" B_{max} value and that the actual B_{max} is close to that of recombinant calreticulin - that is, a value of 83 of moles of Zn^{2+} / moles of protein. Since Zn^{2+} binding to the C-domain of calreticulin is reduced by physiological concentrations of Ca^{2+} and Mg^{2+} we conclude that these Zn^{2+} binding sites may not be utilized under physiological conditions. The C-domain of calreticulin is similar to calsequestrin, a high capacity Ca^{2+} binding protein that was shown to bind over 135 moles Zn^{2+} / mole of protein with low affinity (Ikemoto *et al.*, 1974; Baksh *et al.*, 1995). Calsequestrin, however, does not bind to the Zn^{2+}-IDA sepharose column (Fig. 5-2B). As with the case with the behavior of calsequestrin in the presence and absence of Ca^{2+}, we see yet another difference in the behavior of these two very similar proteins - differential binding of Zn^{2+}. The C-domain of calreticulin was observed to interact *in vitro* with a set of endo(sarco)plasmic reticulum and nuclear proteins (Burns and Michalak, 1993), as well as with the blood clotting factors IX, X and prothrombin (Benedict *et al.*, 1993). The role of Zn^{2+}, if any, in these interactions will require further investigation.

One of the most important findings of this work is identification of a relatively high affinity Zn^{2+} binding site to the N-domain of calreticulin, the region of the protein that is not involved in Ca^{2+} binding (Baksh and Michalak, 1991). We showed that the N+P-domain interacted with Zn^{2+}-IDA-agarose, bound Zn^{2+} as measured using the fluorescence dye SACH and bound $^{65}Zn^{2+}$ under overlay conditions. It is well documented that Zn^{2+} is bound by multiple cysteine and histidine residues (Berg, 1990; Coleman, 1992). All three cysteines and 5 out of 7 histidines found in the mature protein are located in the N-domain of calreticulin (Michalak *et al.*, 1992) (Fig. 5-11). Importantly, these amino acid residues and residues surrounding them are all conserved in calreticulin ranging from higher plants to the human protein (Fig. 1-3) (Michalak *et al.*, 1992; Chen *et al.*, 1994). The remaining two histidines are found in the P-domain of the protein, a region which does not bind Zn^{2+}. Two out of three cysteines found in calreticulin form a disulfide bridge (Cys^{121} and Cys^{146}) (Matsuoka *et al.*, 1994) and, therefore, they are unlikely involved in Zn^{2+} binding to the protein. Therefore, histidine residues in the N-domain may play the most important role in Zn^{2+} binding to calreticulin. Importance of His^{25} is documented by Zn^{2+}-IDA-agarose chromatography of the full length and truncated N+P-domains. The truncated N-domain (missing His^{25}) does not bind very efficiently to the Zn^{2+}-IDA-agarose as compared to the full length domain. DEPC modification of calreticulin further supports the role of histidines in Zn^{2+} binding to the protein. Histidine titration experiments documented that 5 out of 7 histidine residues found in calreticulin may be involved in Zn^{2+} binding to the protein. The remaining two become accessible to

DEPC modification in the absence of Zn^{2+}. Site specific mutagenesis studies should help to establish, in the future, what is the role played by these residues in Zn^{2+} binding to calreticulin.

It is not clear at present why there was a discrepancy between the Zn^{2+} binding studies to the tissue isolated protein and the recombinant protein (Table 5-1). The recombinant protein revealed two classes of binding sites in the presence of 150 mM KCl or 500 mM KCl (the latter condition should kill any false binding arising from electrostatic interactions). A plausible explanation for this discrepancy might be found in calreticulin's interaction with PDI (Chapter Six) and the RNA species (Chapter Three). In the presence of PDI, calreticulin does not interact with a Zn^{2+} chelating column (Fig. 6-1) indicating that PDI may be masking the Zn^{2+} binding within calreticulin. From our PDI interaction studies, PDI was found to interact via the P-domain and possibly the N-domain of calreticulin (Fig. 6-3). The P-domain does not bind Zn^{2+} and the N-domain was found to contain the low capacity site that was observed in the tissue protein. It is therefore possible that the interaction with PDI with the N-domain may therefore mask the Zn^{2+} binding site within the N-domain and hence prevent calreticulin's interaction with the column. The recombinant C-domain was found to interact with the Zn^{2+} chelating column (Fig. 5-6A) and the P+C-domain protein was found to contain only one class of Zn^{2+} binding sites. We therefore concluded that the C-domain contained the high capacity site. If this domain contained the other Zn^{2+} binding site, then in the presence of PDI, the tissue protein should still interact with the column (since the C-domain was observed not to interact with PDI). We, however did not observe this (Fig. 6-1). In the presence of the RNA species, calreticulin was found to interact with the Zn^{2+} chelating column and the RNA species simply flowed through the column. Therefore, the Zn^{2+} binding site was now free to interact with the Zn^{2+} chelating column in the presence of the RNA species. The interaction points for the RNA species has not been determined yet, but based on our knowledge of where the Zn^{2+} binding sites are, it appeared that the RNA interaction site may be within the C-domain of calreticulin (a site not involved in PDI interaction) and therefore this site would be masked in the tissue protein. In the presence of PDI, very little nucleotide signal is observed in the tissue isolated protein. It is therefore possible that both PDI and the RNA molecule may modulate calreticulin's function. Therefore, two possibilities might exist to explain the discrepancy between the Zn^{2+} binding characteristics of the tissue and recombinant proteins: 1) calreticulin might only contain one class of Zn^{2+} binding sites localized to the N-domain (which would therefore be masked in the tissue isolated protein due to the presence of PDI); or 2) calreticulin might contain two classes of Zn^{2+} binding sites with the second class of Zn^{2+} sites localized to the C-domain. However, physiologically, the presence of the second class of Zn^{2+} binding sites (within the C-domain) might be masked by the binding of the RNA molecule or possibly an unknown protein. This would explain the Zn^{2+} binding characteristics of the tissue isolated protein. A detailed anlaysis of the interactions of calreticulin with PDI and the RNA species will enable us to further explain these discrepancies.

The N-domain of calreticulin is one of the most interesting regions of the protein. The amino acid sequence of the N-domain is unique to calreticulin and it is highly conserved among all calreticulins cloned so far (Nash *et al.*, 1994). This region of calreticulin interacts with the DNA binding domain of the glucocorticoid receptor leading to the modulation of the receptor function (Burns *et al.*, 1994). The N-domain of calreticulin may also interact with the cytoplasmic region of the α-subunit of integrin and modulate adhesion-dependent cell signaling (Leung-Hagesteijn *et al.*, 1994). Zn^{2+} may play an important role in the control of these protein-protein interactions. Recently, we have determined that calreticulin (via N- and P-domain) interacts with the protein disulfide isomerase (PDI) in a Zn^{2+}-dependent manner (Chapter Six). Interaction between these two resident ER proteins results in inhibition of the PDI activity and reduction of Ca^{2+} binding to calreticulin.

Little is known about the physiological function of Zn^{2+}-binding proteins. Presence of dual divalent cation binding sites is being discovered in numerous proteins including regulatory proteins such the S100 protein and calmodulin (Baudier *et al*, 1983) and to calreticulin's equivalent in skeletal muscle cells - calsequestrin (Ikemoto *et al.*, 1974; Baksh *et al.*, 1995). The human brain form of calmodulin and S100 were both characterized to have, in addition to two EF hand high affinity calcium binding sites, two zinc binding sites with affinities comparable to those observed for calreticulin. The importance of these proteins still remains obscure but this finding might be able to shed some light into the function of these proteins. Although Zn^{2+} is under homeostatic control, the intracellular distribution of free Zn^{2+} and its possible role in cellular functions is not well established. Increased intracellular concentrations of Zn^{2+} are found in the nucleus, in the synaptic vesicles (Holm *et al.*, 1988), in the sarcoplasmic reticulum membrane subfractions (Picello *et al.*, 1992) and in secretory granules of some cells (Frederickson *et al.*, 1987). These locations are also known to contain calreticulin (Michalak *et al.*, 1992; Johnson *et al.*, 1992; Michalak *et al.*, 1991). Zn^{2+} plays an important role in the regulation of gene expression, action of some metaloenzymes (Berg, 1990; Coleman, 1992). Zn^{2+} has also been shown to block Ca^{2+} influx in some cells (Hide and Beaven, 1991) and induce apoptosis in peripheral blood lymphocytes (Treves *et al.*, 1994). Therefore, it is essential that Zn^{2+} homeostasis is under a precise control at the right place and at the right time. Calreticulin may play an important role in the regulation of the intracellular levels of Zn^{2+} and Ca^{2+}.

CHAPTER SIX

Interaction with Protein disulfide isomerase (PDI)

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INTRODUCTION

Calreticulin (CRT) is localized to the lumen of the ER. Analysis of Zn^{2+} binding to calreticulin lead to the discovery of the interaction with the thiol-disulfide oxidoreductase - protein disulfide isomerase (PDI). PDI is an abundant protein approaching millimolar levels in the ER (Hillson *et al,* 1984). PDI catalyzes the *in vitro* folding of a variety of proteins such as scrambled ribonuclease A (Goldberger *et al,* 1964), scrambled insulin (Tang *et al,* 1988), and certain immunoglobulins (Roth and Pierce, 1987). Mammalian PDI is a homodimer of identical subunits of relative molecular mass 57 000 and pI of 4.1 - 4.5 (Gething *et al,* 1992). Its role in thiol:disulfide exchange reactions may not be its only function. PDI has been found to be highly homologous to the ß subunits of prolyl-4-hydroxylase (Freedman *et al,* 1989), the glycosylation site binding protein (Geetha-Habib *et al,* 1988) and to the thyroid hormone binding protein (Obata *et al,* 1988). PDI has only been shown to be associated with the multi-subunit triglyceride transfer protein complex of liver microsomes (Wetteran *et al,* 1990). Similar to calreticulin, PDI is a resident protein of the ER by virtue of its carboxyl terminal ER retention signal KDEL (Pelham, 1989). In this chapter, we have investigated the interaction between calreticulin and PDI, and determined how this interaction may modulate the physiological function(s) of these two resident ER proteins.

RESULTS

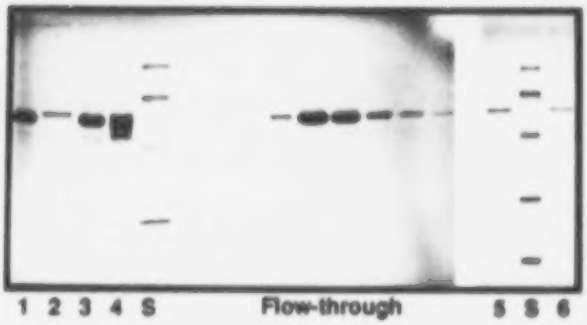
Zn^{2+} Chelating Chromatography of Calreticulin - Interactions of recombinant calreticulin with Zn^{2+} was investigated by Zn^{2+} chelating chromatography in IDA-agarose (Fig. 5-2A). Recombinant calreticulin bound to the Zn^{2+}-IDA-agarose and was eluted with an increasing gradient of imidazole concentration. Calreticulin eluted at approximately 22 mM imidazole (Fig. 5-2A) confirming that the protein interacted with Zn^{2+} under native conditions. We were surprised, however, to find that the native calreticulin, isolated from canine pancreas (Baksh *et al.*, 1992) failed to bind to the Zn^{2+}-IDA-agarose and was found in the column "flow-through" (Fig. 6-1A). The identity of calreticulin in the column "flow-through" was confirmed using specific goat anti-calreticulin antibodies (Fig. 6-1C, lanes 1, 2 and 3) and by the NH_2-terminal amino acid sequence analysis. The goat anti-calreticulin antibody recognized the P- and C-domain of the protein (Fig. 6-1C, lanes 7 and 8). The NH_2-terminal amino acid sequence of the protein found in the "flow-through" fraction was E-P-A-I-Y-K. This sequence was identical to the NH_2-terminal amino acid sequence of calreticulin (Michalak *et al.*, 1992). These results suggested that another protein may have masked the site(s) in calreticulin involved in interaction with Zn^{2+}-IDA-agarose. When an imidazole gradient was applied to the column a protein with a molecular weight of approximately 58 000 eluted at 33 mM imidazole (Fig. 6-1A, lanes 5 and 6). The NH_2-terminal amino acid sequence of this protein band was A-P-E-E-D. This sequence corresponded exactly to the NH_2-terminal amino acid sequence of PDI (Edman *et al.*, 1985). Identity of this

Figure 6-1: SDS-PAGE of Zn^{2+}-IDA Agarose Purified Calreticulin and PDI

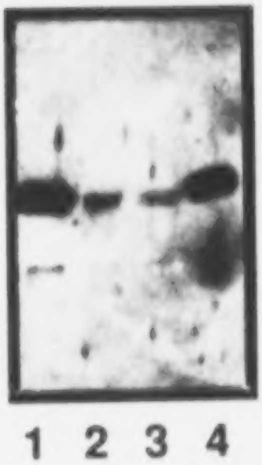
(A) Tissue purified, dog pancreatic calreticulin was separated on a Zn^{2+}-IDA agarose column, equilibrated with 50 mM NaH_2PO_4, pH 7.0 and 100 mM NaCl followed by washing with the same buffer (flow-through) and elution with a linear imidazole gradient (0-50 mM) (lanes 5 and 6) as described under "Chapter Two: Experimental Procedures". One-milliliter fractions were collected and analyzed by SDS-PAGE followed by staining with Coomassie blue. Lane 1, purified calreticulin; lane 2, purified PDI; lane 3 and 4, tissue purified calreticulin; lane 5 and 6, fraction bound to the column and eluted with imidazole gradient (these fractions contain PDI); S, low molecular weight Bio-Rad standards; Flow-through, fractions 1-6 of the column wash (unbound material containing calreticulin). S, Bio-Rad low molecular weight markers - phosphorylase b (97 400), bovine serum albumin (66 200), ovalbumin (42 700), bovine carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500).

(B, C, D) Unbound material (see "flow-through" fractions), bound and eluted fractions (see (A) lanes 5 and 6) were tested for reactivity with the rabbit anti-PDI antibody (B), and with the goat anti-calreticulin antibody (C), and for binding of $^{45}Ca^{2+}$ (D). In B, lane 1, bovine liver PDI; lane 2, native, pancreatic calreticulin containing some PDI; lane 3 "flow-through" fractions from Zn^{2+}-IDA column; lane 4, bound and eluted fractions from Zn^{2+}-IDA column. In C, lane 1, pancreatic calreticulin; lane 2, recombinant calreticulin; lane 3, "flow-through" fractions from Zn^{2+}-IDA column; lane 4, bovine liver PDI; lane 5, bound and eluted fractions from Zn^{2+}-IDA column; lane 6, GST-N-domain fusion protein; lane 7, GST-P-domain fusion protein; lane 8, GST-C-domain fusion protein. In D, proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes and incubated with $^{45}Ca^{2+}$; lane 1, "flow-through" fraction from Zn^{2+}-IDA column (contains calreticulin); lane 2, bound and eluted fraction from Zn^{2+}-IDA (contains PDI).

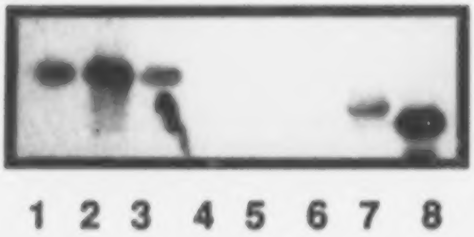
A


1 2 3 4 S
Flow-through
5 S 6

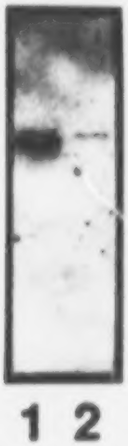
B



C



D



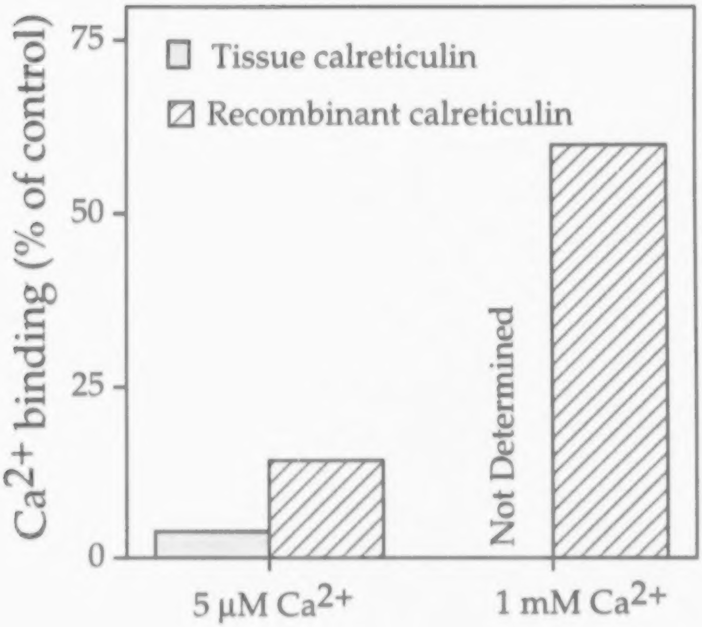
eluted protein band to PDI was further confirmed using specific anti-PDI antibodies (Fig. 6-1B, lane 4). A small degree of cross-reactivity was observed with the anti-PDI antibodies in the column "flow-through"(Fig. 6-1B, lane 3); no protein immunoreactive against goat anti-calreticulin was observed in the eluted PDI fractions (Fig. 6-1C, lane 5), further confirming the separation of these two proteins using Zn^{2+}-IDA-agarose. In Fig. 6-1D, the calreticulin found in the Zn^{2+}-IDA-agarose "flow-through" bound large quantities of $^{45}Ca^{2+}$ (lane 1), while PDI bound much lower quantities of $^{45}Ca^{2+}$ as compared to calreticulin (Fig. 6-1D compare lanes 1 and 2). We concluded that calreticulin and PDI may interact and this interaction may prevent calreticulin binding to the Zn^{2+}-IDA-agarose.

Ca^{2+} Binding to Calreticulin In the Presence of PDI - Recombinant or native calreticulin have two Ca^{2+} binding sites; a high capacity site (>25 moles / mole of protein) and a high affinity Ca^{2+} binding site ($K_d \sim 4 - 10\ \mu M$) (Ostwald and MacLennan, 1974; Baksh and Michalak, 1991). In the presence of PDI Ca^{2+} binding to the high affinity site in calreticulin was dramatically reduced (Fig. 6-2 at 5 μM Ca^{2+}). This was not surprising as the high affinity Ca^{2+} binding site is localized to the P-domain in calreticulin (Baksh and Michalak, 1991), a site of interaction with PDI (see Fig. 6-3). PDI had no effect on the low affinity and high capacity Ca^{2+} binding (located in the C-domain) to the protein (Fig. 6-2 at 1 mM Ca^{2+}).

PDI Interacted with the P-domain of Calreticulin - We were interested in identification of the specific region of calreticulin involved in the

Figure 6-2: PDI's Effect on Calreticulin's High Affinity Ca^{2+} Binding Site

Ca^{2+} binding in the presence of PDI. Analysis of Ca^{2+} binding to the PDI : Calreticulin (1:1) complex was carried out by equilibrium dialysis as described under "Chapter Two: Experimental Procedures." Bar graph shows binding of $^{45}Ca^{2+}$ to the PDI: Calreticulin complex in a 1:1 molar ratio (average of duplicate runs). A large amount of tissue calreticulin was not available to perform the measurements at 1 mM Ca^{2+}. 100% Ca^{2+} binding was determined in the presence of calreticulin (free of PDI) and was used as the 100% value.

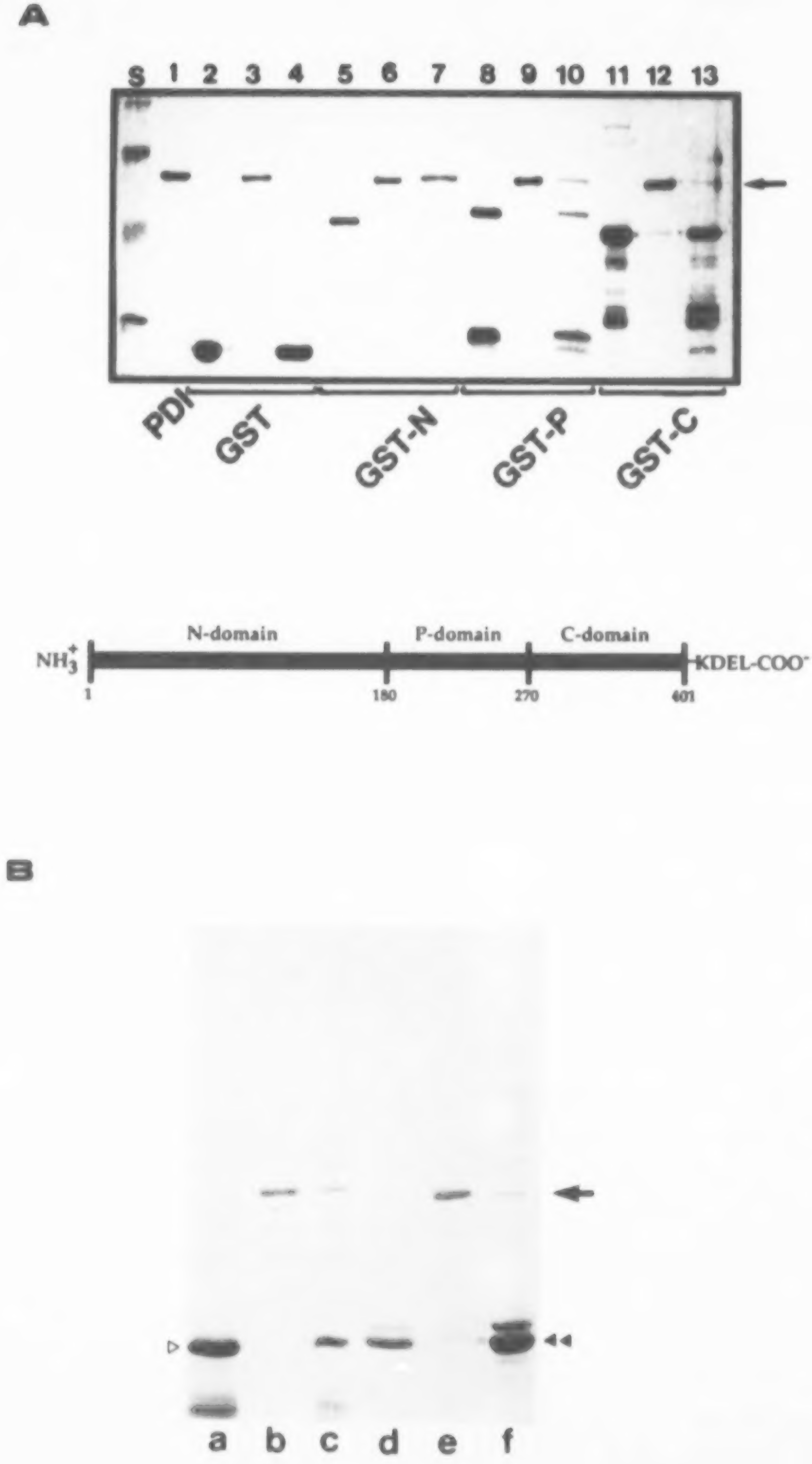

Ca2+ binding (% of control)
75
50
25
0
Tissue calreticulin
Recombinant calreticulin
Not Determined
5 μM Ca2+
1 mM Ca2+

interactions with PDI. In these experiments we have used the GST-calreticulin fusion proteins synthesized in our earlier studies on the identification of Ca^{2+} binding site in calreticulin (Baksh and Michalak, 1991). Three domains of calreticulin were used in this study: the GST-N-domain (encompassing the NH_2-terminal 182 amino acids of the protein), the GST-P-domain (residues 139 to 273) and the GST-C-domain (residues 270 to 401) (Fig. 6-3A). GST was used as a control. GST or the GST-fusion proteins were first bound to the Glutathione-agarose beads followed by the addition of purified PDI and centrifugation to collect the unbound material. The pellet was incubated with 5 mM glutathione to elute proteins bound to the GST-affinity beads. We predict that if PDI interacted with calreticulin domain(s) the two proteins should elute from the beads together as a protein complex. First, we established that PDI did not interact with GST. Figure 6-3A shows that PDI did not bind to the GST-Glutathione-agarose and was found in the unbound fraction (lane 3), whereas the recombinant GST was eluted with 5 mM glutathione (lane 4). We conclude that PDI did not interact with the recombinant GST. When incubated with the GST-N-domain affinity beads, the majority of PDI eluted in the unbound fraction (Fig. 6-3A, lane 6). The remaining PDI and GST-N-domain were eluted with 5 mM glutathione (Fig. 6-3A, lane 7), suggesting that there were limited interactions between GST-N-domain of calreticulin and PDI. When GST-P-domain affinity beads were incubated with PDI, almost all of the PDI and the GST-P-domain eluted together from the column with 5 mM glutathione (Fig. 6-3A, lane 10) indicating that the two proteins interacted. Based on the Coomassie blue stained intensities of these two protein bands we conclude that they

Figure 6-3: PDI Binding to Calreticulin Domains

(A) GST, GST-*N-domain*, GST-*P-domain* and GST-*C-domain* of calreticulin were expressed in *E. coli* and purified as described by Baksh and Michalak (1991). The GST and GST fusion proteins were bound to the Glutathione-agarose beads, washed with phosphate-buffered saline and then incubated with purified PDI as described under "Experimental Procedures". The beads were spun down to obtain the unbound material (*lanes 3, 6, 9*, and *12*) followed by incubation with 5 mM glutathione to obtain the bound fractions (*lanes 4, 7, 10*, and *13*). *Lane 1*, purified PDI; *lane 2*, GST; *lanes 3 and 4*, unbound and bound fractions from the GST column, respectively; *lanes 6 and 7*, unbound and bound fractions from the GST-*N-domain* column, respectively; *lanes 9 and 10*, unbound and bound fractions from the GST-*P-domain* column, respectively; *lanes 12 and 13*, unbound and bound fractions from the GST-*C-domain* column, respectively. *Lane 5*, purified GST-*N-domain*; *lane 8*, purified GST-*P-domain*; *lane 11*, purified GST-*C-domain*. *Arrows* depicts the position of PDI. Schematic representation of calreticulin domains is shown below in the figure.

(B) GST-*HA3* (containing the first two repeat sequence A repeats) and GST-*HA10* (containing the last two repeat sequence A repeats) were expressed in *E. coli* and purified as described by Baksh and Michalak (1991). The experimental protocol in *A* was repeated for these fusion proteins in order to determine if the repeat sequences may be involved in interaction with PDI. *Lane a*, purified GST-*HA3*; *lanes b and c*, unbound and bound fractions from the GST-*HA3* column, respectively; *lane d*, purified GST-*HA10*; *lanes e and f*, unbound and bound fractions from the GST-*HA10* column, respectively. *Arrow* indicates position of PDI; *open triangle* indicates position of GST-*HA3*, and the *closed triangle* the position of GST-*HA10*.


A
S
1
2
3
4
5
6
7
8
9
10
11
12
13
PDI
GST
GST-N
GST-P
GST-C
N-domain
P-domain
C-domain
NH$_3^+$
KDEL-COO$^-$
1
180
270
401
B
a
b
c
d
e
f

interacted with 1:1 ratio. Figure 6-3A showed that the GST-*C-domain* of calreticulin did not interact with PDI as >90% of the PDI eluted alone in the unbound fraction (*lane 12*), followed by elution of the GST-*C-domain* with 5 mM glutathione (*lane 13*). We conclude that PDI interacts tightly with the *P-domain* of calreticulin and to a lesser extent with the *N-domain* of the protein.

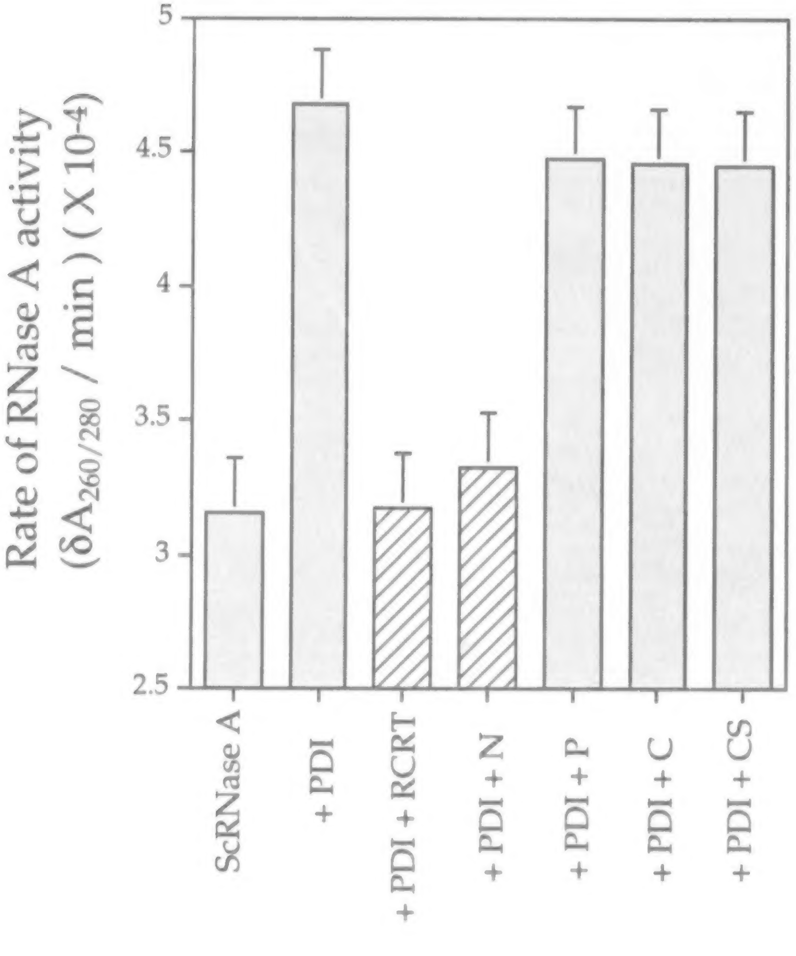
To further elucidate how PDI may be interacting with the *P-domain* of calreticulin, two truncated *P-domain* fragments (GST-*HA3* and GST-*HA10*, Fig. 4-7A) were used in similar affinity chromatography experiments as described above. GST-*HA3* (~ 32 kDa) is missing the last repeat sequence A (Fig. 1-2B) and GST-*HA10* (~ 34 kDa) is missing the first repeat sequence A. These GST-fusion proteins were first bound to the Glutathione-agarose beads followed by the addition of purified PDI and centrifugation to collect the unbound material. The pellet was incubated with 5 mM glutathione to elute proteins bound to the GST-affinity beads. We predict again that if PDI interacted with calreticulin domain(s) the two proteins should elute from the beads together as a protein complex. When incubated with the GST-*HA3* affinity beads, the majority of PDI eluted in the unbound fraction (Fig. 6-3B, *lane b*). The remaining PDI and GST-*HA3* were eluted with 5 mM glutathione (Fig. 6-3B, *lane c*), suggesting that there were limited interactions between GST-*HA3* and PDI. This truncated *P-domain* fragment (missing the last repeat sequence A) interacted with PDI to a lesser extent than the full length *P-domain* sample (compare Fig. 6-3A, *lane 10* with Fig. 6-3B, *lane c*). When GST-*HA10* affinity beads were incubated with PDI, once again we observed that the majority of PDI eluted in the unbound fraction (Fig. 6-3B, *lane e*).

The remaining PDI (< 5 %) and the GST-HA10 protein were eluted with 5 mM glutathione (Fig. 6-3B, lane f), suggesting that there were limited interactions between GST-HA10 and PDI. This truncated P-domain fragment (GST-HA10 - missing the first repeat sequence A) interacted with PDI to a lesser extent than the GST-HA3 sample (containing the first repeat sequence A) (compare Fig. 6-3B, lane c with Fig. 6-3B, lane e). We concluded that both the first and third repeat sequence A repeat segments (and more importantly the first repeat sequence A) within the P-domain of calreticulin may be responsible for the interaction with PDI.

The Effect of Calreticulin on PDI Activity - In order to test the ability of calreticulin and calreticulin domains to affect PDI function, PDI activity was measured as the ability of the protein to assist in refolding of scrambled RNase A (Noiva and Lennarz, 1992; Freedman et al., 1994). Scrambled RNase A was incubated in the presence and absence of PDI and PDI complexed with calreticulin and calreticulin domains or calsequestrin as a control. Figure 6-4 described the ability of PDI to refold scrambled RNase A in the presence of calreticulin or calreticulin domains. A plot of the rate of RNase A activity is shown. In the presence of PDI, the recovery of RNase activity was significantly enhanced (Fig. 6-4). This effect of PDI was drastically reduced in the presence of calreticulin (Fig. 6-4 - + RCRT), and the N-domain (Fig. 6-4 - + N). In the presence of the P-domain, RNase A activity 96 % that of control (that is, with PDI) indicating that this domain does not affect PDI activity. Both the C-domain (+ C) of calreticulin and calsequestrin (+ CS) had no significant affect on PDI activity. Since the N-domain interacted with PDI

Figure 6-4: The Effect of Calreticulin and Calreticulin Domains on PDI Activity

Recovery of ribonuclease activity of the scrambled RNase A (ScRNase A) was measured in the presence of PDI (+PDI), recombinant calreticulin (+ RCRT), the N-domain of calreticulin (+N), the P-domain of calreticulin (+P), the C-domain of calreticulin (+C), and canine cardiac calsequestrin (+CS) as described under "Chapter Two: Experimental Procedures". The $A_{260/280}$ ratio was measured to determine the extent of cleavage of yeast RNA by re-folded ScRNase A. 1: 1 molar ratio of test sample : PDI was used. Results (of triplicate runs), indicating the rate of change of the $A_{260/280}$ over time, were obtained from the slope of plots of the change of $A_{260/280}$ over a period of 60 minutes. Hatched boxes indicate the samples significantly inhibiting the ability of PDI to refold scrambled RNase A ($P < 0.006$, $n = 3$, student's t-test).


Rate of RNase A activity
($\delta A_{260/280}$ / min) (X 10^{-4})
5
4.5
4
3.5
3
2.5
ScRNase A
+ PDI
+ PDI + RCRT
+ PDI + N
+ PDI + P
+ PDI + C
+ PDI + CS

(Fig. 6-3A, lane 12 and 13) and affected PDI activity; and the P-domain interacted with PDI (Fig. 6-3A, lane 4) but did not affect PDI activity, we concluded that PDI might make two contact points within calreticulin - the N-domain and the P-domain. PDI interacted tightly with the P-domain of calreticulin, but did not affect its isomerase activity; but PDI interacted to a lesser extent with the N-domain of the protein which drastically reduced its isomerase and/or refolding activity (Fig. 6-4).

DISCUSSION

In this chapter we have discovered that calreticulin interacted with PDI in a Zn^{2+}-dependent manner. PDI interacted with the P-domain and partially with the N-domain of calreticulin and reduced the high affinity Ca^{2+} binding to the protein. Calreticulin binding to PDI inhibited PDI activity as measured by refolding of the scrambled RNase A.

The PDI/calreticulin complex can be broken up by the Zn^{2+}-IDA chromatography suggesting that these interactions may be Zn^{2+}-dependent. In the presence of PDI calreticulin did not bind to the Zn^{2+}-IDA-agarose. PDI must therefore interact with calreticulin at or near its Zn^{2+} binding site(s) or induced conformational changes within the protein thus preventing interaction of calreticulin with the Zn^{2+}-IDA-agarose. Calreticulin and PDI are Ca^{2+} binding proteins of the ER lumen (Michalak *et al.*, 1992; Lebeche *et al.*, 1994). Several other ER lumenal proteins, including ERp72, reticulocalbin, Grp94 (endoplasmin, ERp99), and BiP (Grp78), may also play an important role in Ca^{2+} binding and storage within the ER membrane (Koch, 1987 and 1990; Mazzarella *et al.*, 1990; Mazzarella and Green, 1987; Milner *et al.*, 1992; Ozawa and

Muramatsu, 1993; Lucero *et al.*, 1994; Racchetti *et al.*, 1994). Many of these proteins are involved in the posttranslation modification/folding of newly synthesized secretory proteins. Their retention in the lumen of ER is dependent on the KDEL carboxyl-terminal amino acid sequence (Pelham, 1989). Koch (1987) proposed the name reticuloplasmin for the ER lumenal proteins to emphasize their specific location and to consider their possible role in ER structure and function including storage of Ca^{2+}. Of these proteins, PDI and calreticulin were considered the two proteins accounting for the major proportion of the Ca^{2+} binding activity (Macer and Koch, 1988). Interactions between calreticulin and PDI reported here support this hypothesis and show that these two reticuloplasmins interact. Importantly, these interactions may modulate function of these ER lumenal proteins.

Interactions between calreticulin and PDI are supported by recent studies by Nigam *et al.* (1994). While studying a set of ER chaperones these authors isolated a complex of ER resident proteins that bind to denatured protein columns in an ATP and Ca^{2+}-dependent manner. These proteins were identified as BiP (Grp78), Grp94, calreticulin, PDI, ERp72, p50 and 46-kDa protein. Direct interaction between calreticulin and denatured proteins was not reported. It is possible that calreticulin binds to the denatured proteins *via* interaction with PDI as described here. This hypothesis is supported by the finding that PDI interacts with calreticulin as determined by calreticulin affinity chromatography (Baksh et al. 1994 Zn^{2+} binding to calreticulin and interaction with Protein Disulfide Isomerase. J. Biol. Chem., *submitted*). The interaction between PDI and calreticulin is ion dependent.

PDI is an abundant protein approaching millimolar levels in the lumen of ER (Noiva and Lennarz, 1992; Freedman *et al.*, 1994). The protein catalyzes the *in vitro* folding of a variety of proteins by assisting in isomerization of intramolecular disulfide bridges (Noiva and Lennarz, 1992; Freedman *et al.*, 1994). Calreticulin and PDI have both been proposed to be multifunctional proteins capable of interaction with a variety of proteins (Michalak *et al.*, 1992; Noiva and Lennarz, 1992; Freedman, 1994; Burns *et al.*, 1994). In addition to their ion binding and protein binding activities, both proteins (or their homologues) have been implicated to modulate gene expression (Johnson *et al.*, 1992; Burns *et al.*, 1994; Dedhar *et al.*, 1994), were identified as autoimmune antigens (McCauliffe *et al.*, 1990b; Yokoi *et al.*, 1993) and were found complexed with the intracellular lipid droplets (Ghosal *et al.*, 1994). Importantly, this study supports the aforementioned observations for the interaction between calreticulin and PDI and the effect that this interaction has on functions of these two resident ER proteins. In the presence of PDI calreticulin does not bind Ca^{2+} with high affinity, whereas PDI shows significantly reduced ability to refold scrambled RNase. It is tempting to speculate that calreticulin/PDI while complexed may perform additional functions likely involved in posttranslational modification of the newly synthesized proteins.

We have determined that PDI interacted with the P-domain and to a lesser extent with the N-domain of calreticulin (Fig. 6-5). Importantly, calreticulin's interaction with PDI was reduced when either the first and

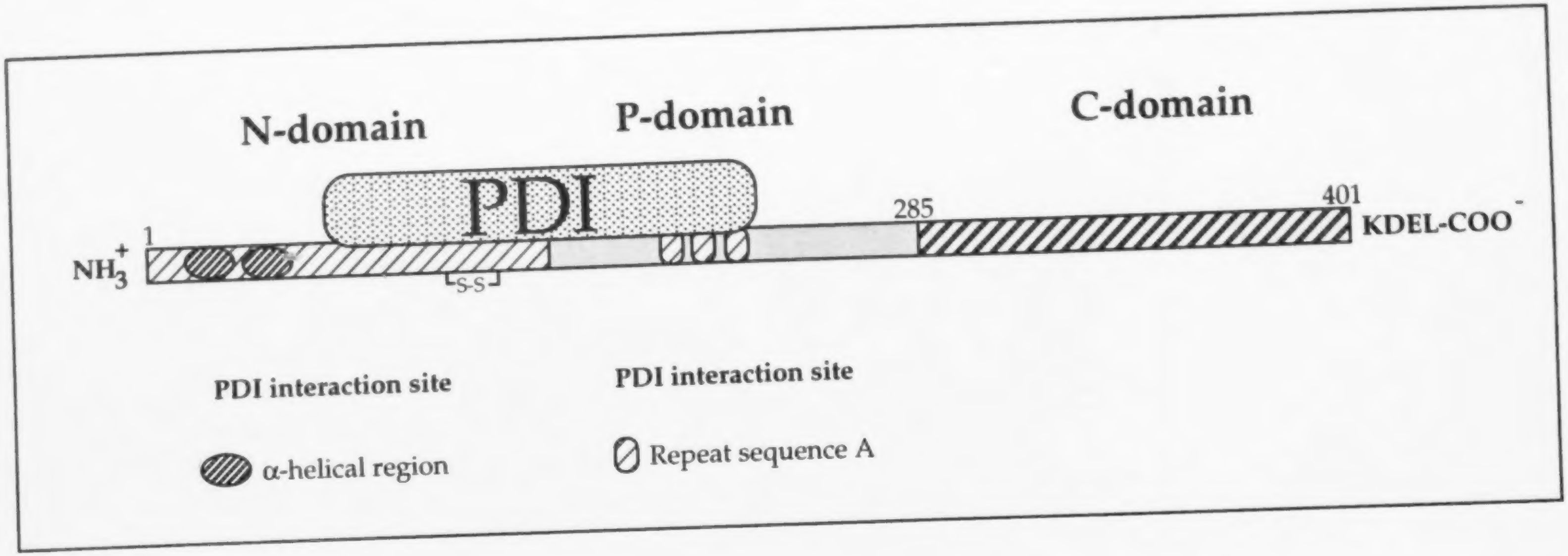

N-domain
P-domain
C-domain
PDI
1
285
401
NH$_3^+$
KDEL-COO$^-$
S-S
PDI interaction site
PDI interaction site
α-helical region
Repeat sequence A

Figure 6-5: Calreticulin domains involved in potential interaction with PDI

Putative location of the interaction points with PDI - the N-domain and P-domain are indicated. Numbers indicate amino acid position within rabbit skeletal muscle calreticulin sequence.

third repeat sequence A repeat segments was removed (Fig. 6-3B). Both of these repeat segments were determined to be involved in high affinity Ca^{2+} binding to calreticulin (Fig. 4-6C and 4-8C). It is not surprising, therefore, that PDI inhibited binding of Ca^{2+} to the high affinity site in calreticulin. No significant effects of PDI on the high capacity Ca^{2+} binding site (localized to the C-domain) of calreticulin was observed. It has been shown by de Jong *et al.* (1991) that polyproline inhibits chicken prolyl hydroxylase with increasing length of the peptide substrate. PDI is highly homologous to the ß component of prolyl hydroxylase (Freedman *et al*, 1989), and the P-domain of calreticulin contains 16 mole % prolines and each of the repeat sequence A segments contain three proline residues. Hence, it might be possible that the interaction between PDI and calreticulin might involve the abundance of proline residues within the high capacity Ca^{2+} binding site. Therefore, binding of PDI to calreticulin influenced a function of the central portion of the molecule. It was also shown that the N-domain (containing the high affinity Zn^{2+} binding site) of calreticulin inhibited the ability of PDI to refold scrambled RNase A. PDI may therefore make two contact points within calreticulin - the N-domain and the P-domain (Fig. 6-5). PDI is a homodimer of identical subunits (Gething *et al.*, 1992) and may thus require two contact points within calreticulin to stabilize the interaction between these two proteins. Further experiments will have to be conducted to determine the exact contact points between these two resident ER proteins.

It thus appeared that all three domains of calreticulin are involved in protein-protein interactions: the N-domain interacts with the DNA binding domain of glucocorticoid receptor (Burns *et al.*, 1994) and possibly

PDI (this study), the P-domain also interacts with PDI (this study), and the C-domain interacts with a set of small molecular weight ER membrane proteins (Burns and Michalak, 1993) and Factor IXa (Kuwabara *et al.*, 1995). The P-domain of calreticulin has a repeat sequence (the repeat sequence A) which may be involved in the high affinity Ca^{2+} binding to the protein. A similar amino acid sequence is found in calnexin (Bergeron *et al.*, 1994) and in calmegin, a Ca^{2+} binding protein specifically expressed during male meiotic germ cell development (Ohsako *et al.*, 1994; Watanabe *et al.*, 1994). Similar to calreticulin, the P-domain region in calnexin binds Ca^{2+} (Tjoelker *et al.*, 1994). It is conceivable that both calnexin and calmegin may also interact with PDI and their Ca^{2+} binding properties may be modified by these interactions.

CHAPTER SEVEN

Summary and Future Directions

SUMMARY OF THESIS

In this thesis I have shown that: (i) two distinct Ca^{2+} binding sites are present within calreticulin that together coordinate 20 - 22 moles of Ca^{2+} per mole of calreticulin. These sites can be divided into a high affinity, low capacity site localized within the proline-rich P-domain of calreticulin and the low affinity, high capacity sites within the highly acid-rich C-domain of the protein; (ii) two distinct Zn^{2+} binding sites are present within recombinant calreticulin that together coordinate >100 moles of Zn^{2+} per mole of calreticulin. These sites can be divided into the high affinity, low capacity sites within the highly globular N-domain of calreticulin and the intermediate affinity, high capacity sites within the highly charged C-domain of the protein. However, tissue isolated calreticulin was observed to contain only the low capacity site located within the N-domain; (iii) Mg^{2+} influences both the high capacity Ca^{2+} binding and Zn^{2+} binding sites, but does not influence the high affinity Ca^{2+} binding or Zn^{2+} binding sites indicating that latter sites could be functionally significant to the protein; (iv) calreticulin interacts with an ER resident protein, protein disulfide isomerase in a Zn^{2+}-dependent manner via its N-domain and P-domain. PDI drastically reduced high affinity Ca^{2+} binding to the P-domain and calreticulin inhibited PDI's ability to refold scrambled RNase A.

These results support the multifunctional characteristic of calreticulin and the different roles it could play in the normal functioning of the cell. Calreticulin's domain-like organization may allow it to carry out diverse functions that include ion binding and protein interactions (Fig. 7-1).

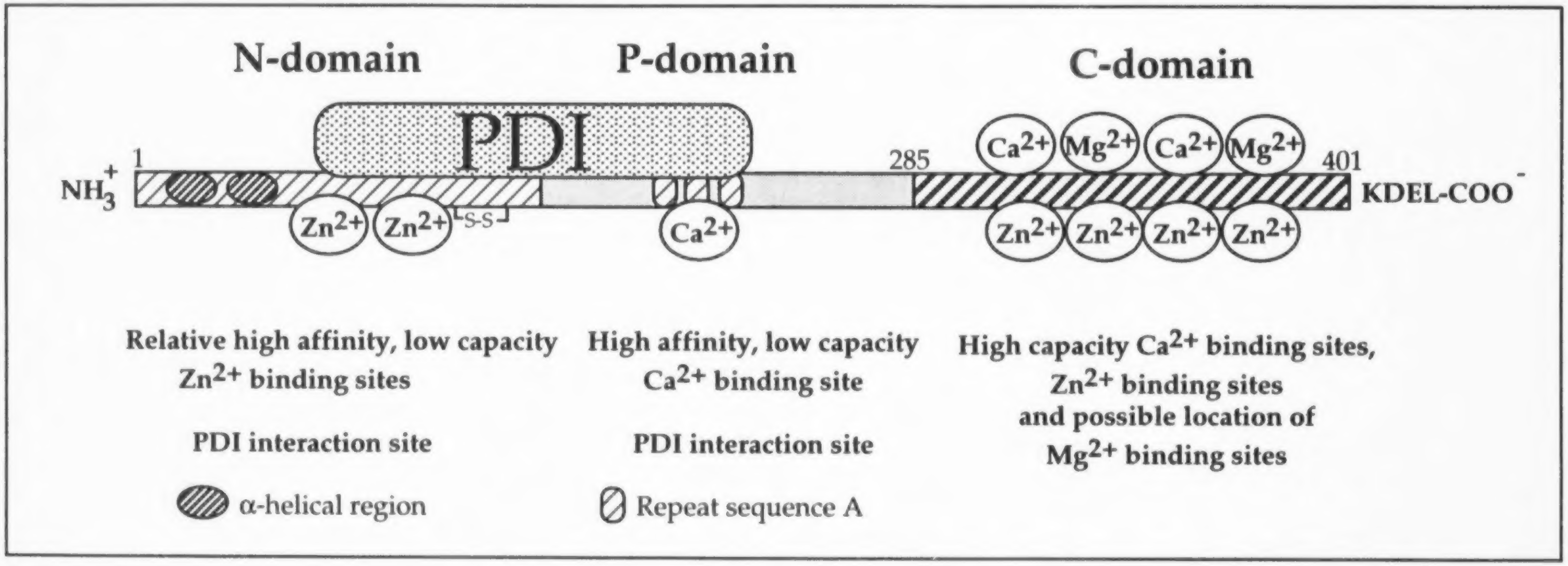

N-domain
P-domain
C-domain
PDI
1
285
401
NH3+
KDEL-COO-
Zn2+
Zn2+
S-S
Ca2+
Ca2+
Mg2+
Ca2+
Mg2+
Zn2+
Zn2+
Zn2+
Zn2+
Relative high affinity, low capacity Zn2+ binding sites
PDI interaction site
α-helical region
High affinity, low capacity Ca2+ binding site
PDI interaction site
Repeat sequence A
High capacity Ca2+ binding sites, Zn2+ binding sites and possible location of Mg2+ binding sites

Figure 7-1: Calreticulin domains involved in ion binding and interaction with PDI

Putative location of the interaction points with ions and PDI are indicated.

Numbers indicate amino acid position within rabbit skeletal muscle calreticulin sequence.

Importance of the Ca^{2+} binding sites?

The levels of Ca^{2+} within living cells fluctuate between the external pool, the cytosolic fraction, and that sequestered by internal orangelles. Calreticulin was found to be the major Ca^{2+} binding protein of smooth muscle SR and non-muscle ER (Milner *et al.*, 1991). Calreticulin's homologue within skeletal muscle, calsequestrin, was found to be localized to the cisternae of the SR and to be responsible for buffering Ca^{2+} levels within the SR (MacLennan, 1972; Cala and Jones, 1983). The high amount of Ca^{2+} bound to the C-domain of calreticulin would certainly support a Ca^{2+} storage role for the protein. Based on a comparison between the free Ca^{2+} concentration and total Ca^{2+} concentration within squid axons, it appears that less than 0.1% of the total Ca^{2+} is free (Thomas, 1982). The rest is sequestered by Ca^{2+} binding proteins within Ca^{2+} sequestering organelles, namely the mitochondria and the sarco(endo)plasmic reticulum. Calreticulin has been found in many of the organs where there is a high oscillation of Ca^{2+} and hence there would be a requirement for a buffer for Ca^{2+}. These include the SR of smooth muscle cells and the ER of non-muscle cells (Milner *et al.*, 1991); the neurons of the marine mollusk, *Aplysia californica* (Kennedy *et al.*, 1992); the acrosome body of spermatozoa and spermatids (Nakamura et al., 1992a); in the penetration of the skin by cercariae of *Schistosoma mansoni* (Davies, 1983; Khalife *et al.*, 1994); in the blood coagulation cascade (Rapaport and Roa, 1992; Sueyoshi *et al.*, 1991); and in the killing activity of perforin molecules (Dupuis *et al.*, 1993). Calreticulin has also been found to be possibly involved in a autoimmune disorders including systemic lupus erythematosus (SLE) (Lieu *et al.*, 1988), Long Evans

Syndrome (LEC) (Yokoi *et al.*, 1993), and a new human rheumatic disease autoantigen that shares an intimate relationship with the Ro/SS-A ribonucleoprotein (Malhotra *et al.*, 1993). It was found by Odink *et al.* (1987) that two Ca^{2+} binding proteins are expressed in macrophages of rheumatic arthritis that have been implicated in macrophage activation (Hartung, 1983), and the expression of these two proteins and their interactions with other proteins could be important in the regulation of macrophage differentiation. Calreticulin may provide the Ca^{2+} that is needed for the functioning of these important events.

A function for calreticulin's high affinity site within the P-domain still remains to elucidated. As discussed in Chapter Four, calreticulin does not have an EF-hand high affinity Ca^{2+} binding sequence that is usually found in high affinity Ca^{2+} binding proteins such as calmodulin or troponin C (Kretsinger, 1988). Binding only 1 mole of Ca^{2+} per mole of protein would imply a regulatory role for this high affinity site. All of the processes mentioned above require a large amount of Ca^{2+} that may be supplied by calreticulin's high capacity Ca^{2+} binding site within the C-domain of the protein. What this regulatory role is still remains to be determined. Calreticulin has been found to be transiently associated with milk lipid droplets during lactation (Ghosal *et al.*, 1994) and to be associated with PDI via it's P-domain (Chapter Six). PDI affects Ca^{2+} binding to the high affinity site within the P-domain. It still has to be determined which part of calreticulin interacts with lipid droplets during lactation. It would be curious to investigate if this association is Ca^{2+} dependent and if it may control the transient nature of its association with lipids.

Importance of the Zn^{2+} binding sites?

The mechanisms governing cellular uptake and release of this most abundant intracellular heavy metal has not been elucidated as yet, although the intracellular concentration of this ion has been shown to rapidly change in response to a variety of physiological factors (Pattison and Cousins, 1986). As in the case of intracellular Ca^{2+}, 10% of the total intracellular Zn^{2+} concentration is free to take part in transient cellular processes (Treves *et al.*, 1994). The rest is stored and is only released in response to extracellular processes. Calreticulin was found to bind an enormous amount of Zn^{2+} (Chapter Six). Two population of binding sites were found - a high capacity site within the C-domain and a high affinity site within the N-domain of the protein. The high capacity Zn^{2+} site, like the high capacity Ca^{2+} binding site may serve a storage function. Calreticulin has been found in neuronal tissues and to be associated with the IP_3R/Ca^{2+} channel (Treves *et al.*, 1992; Payrs *et al.*, 1994). Zn^{2+} has also been found to be actively taken up and stored in synaptic vesicles in nerve terminals, and stimulation of nerve fibre tracts that contain large amounts of Zn^{2+} can induce Zn^{2+} release, suggesting that it may act as a neuromodulator (Xie and Smart, 1991; Assaf and Chung, 1984). The storage for the estimated 200 - 300 µM of Zn^{2+} within synaptic vesicles has not be established yet (Federickson, 1989). It is possible that the C-domain may account for this buffering of Zn^{2+} within neuronal cells. Even at physiological concentrations of Mg^{2+}, calreticulin still has 50% binding of Zn^{2+} at its high capacity site (~ 40 moles Zn^{2+}/mole of protein; see Chapter Six). Apart from this possible storage function, no other physiological function can be assigned to this Zn^{2+} binding site.

The role of the high affinity Zn^{2+} binding site may be observed in calreticulin's interaction with the glucocorticoid receptor (Burns *et al.*, 1994) and in calreticulin's involvement in dexamethosome induced apoptosis (Burns, Michalak, Bleackley, unpublished results). The glucocorticoid receptor requires Zn^{2+} to properly form its Zn^{2+}-finger motif enabling it to interact with its DNA response element within the nucleus. Calreticulin, in it's interaction with the receptor may provide the Zn^{2+} requirement for this transcriptional control event.

Apoptosis or programmed cell death is an important process in embryogenesis, lymphocyte negative selection in the thymus, immune-mediated responses and other events involving the immune system (Martin *et al.*, 1994). Calreticulin has also been shown to be a component of cytotoxic T cells and to be present in elevated levels in mitogen-stimulated T cells (Burns *et al.*, 1992; Dupuis *et al.*, 1993). Treves *et al.* (1994) elegantly showed that apoptosis (the end event of cytotoxic T cell action) is dependent on intracellular Zn^{2+} and independent on intracellular Ca^{2+} in lymphocytes. Calreticulin could be involved in both processes of cytotoxic T cell action - Ca^{2+} mediated killing of cells via its Ca^{2+} binding storage site and interaction with perforin; and the removal of Zn^{2+} within the cells the T cell is attacking and the storage of Zn^{2+} within the high capacity Zn^{2+} site of the C-domain - a situation known to induced apoptosis (Treves *et al.*, 1994. A more detailed analysis of calreticulin and its Zn^{2+} binding site in programmed cell death will have to be conducted to fully understand its role in controlling T cell activities.

Zn^{2+} has also been shown to be involved in the acrosome formation in sea urchin sperm (Clapper *et al.*, 1985) and calreticulin has been intensely investigated as being involved in acrosome formation and sperm-egg fusion activities (Nakamura *et al.*, 1993; Nakamura *et al.*, 1992a; Nakamura *et al.*, 1992b). Clapper *et al.* (1985) found that low Zn^{2+} concentrations (0.1 μM) promoted sperm motility and acrosome reaction, but high Zn^{2+} concentrations (10 - 30 μM) inhibited acrosome reactions. A tight control of the amount of Zn^{2+} is thus required for this pre-fusion event. The presence of calreticulin in the pre-acrosome and then in the acrosome of spermatocytes and spermatids would favor calreticulin being a candidate for controlling the concentration of Zn^{2+} available for the acrosome reaction. The association constants for Zn^{2+} binding to the N-domain and C-domain are well within the ranges for this kind of control mechanism. Once the acrosome body has formed, calreticulin's Ca^{2+} binding function may come into play providing the Ca^{2+} for the sperm-egg fusion event. Nakamura *et al.* are currently testing these hypotheses.

Calreticulin has been shown to be the homologue of a rat duodenal iron-binding protein mobilferrin (Conrad *et al.*, 1993). Mobilferrin is thought to be responsible for the removal of iron into nonintestinal cells that do not contain transferrin receptors (which normally functions in the uptake of iron). This alternate iron transport pathway is only partially shared with Zn^{2+} and Cd^{2+} (Conrad *et al.*, 1994) and was found to utilized integrin molecules (Conrad *et al.*, 1994). Presence of Zn^{2+} binding sites within calreticulin would support a role in the function of this protein in Zn^{2+} transport via the alternate iron transport pathway in nonintestinal cells. Again one has to debate the cytoplasmic localization of this protein to perform this transport function. But, Conrad *et al.* (1993a; and 1990) clearly showed that mobilferrin has the same NH_2-

terminus of calreticulin, similar behavior on SDS-PAGE and reactivity to sera against Ro/SS-A patients (Conrad *et al.*, 1991), and similar Zn^{2+} binding characteristics. It thus appears that calreticulin, when outside its natural environment of the ER is engaged in various new aspects of cellular function.

Is calreticulin an RNA binding protein?

Calreticulin has been found to interact with two varied RNA molecules - the Ro/SS-A hY RNA molecule (Deutscher *et al., 1988*) and the positive strand of the rubella viral (RV) RNA molecule (Singh *et al.*, 1994). Calreticulin has also been found to undergo cell surface localization upon cytomegaloviral induction of human fibroblasts (Kawashima *et al.*, 1994). Both the hY and RV RNA molecules exist in the cytosol and were found to interact with calreticulin in the cytosol. Neither of these groups have tested the effect of divalent cations on the association of calreticulin with the respective RNA molecules. Certain preparations of tissue calreticulin were found to contain large amounts of as yet unidentified 60 - 100 nucleotide RNA molecule. Separation was only attainable on a Zn^{2+}-IDA column. Furthermore, it was also observed that when PDI was found to be associated with calreticulin, the RNA molecule was present a very low to undetectable amounts. The PDI and RNA binding sites might be in close association within each other within calreticulin. The identity of this unknown RNA molecule may enlighten calreticulin's function within the cell.

Is it another chaperone originating from the ER?

The ER is the initial site in the secretory pathway where membrane and secretory proteins are folded and assembled. Many of the resident proteins within the lumen of the ER have been shown to carry out chaperone-associated functions within the cell (Nigam *et al.*, 1994; Pelham, 1991). Nigam *et al.* (1994) showed that their might be an ATP and Ca^{2+} requirement for the association of Grp94, protein disulfide isomerase, ERp72 and calreticulin with a variety of denatured proteins, including histone, gelatin, α fetoprotein, thyroglobulin, lysozyme, and casein. These proteins appear to function as Ca^{2+}-dependent chaperones, with ATP (10^{-6} M) required for dissociation of the complex of chaperone/denatured protein (Rothman, 1988). Calreticulin's Ca^{2+} binding ability would support calreticulin's role as a molecular chaperone. However, calreticulin's lack of an ATP binding site would disagree with its ability to be autophosphorylated and hence in it's ATP regulation in the proposed chaperone associations in the experiments of Nigam *et al.* (1994). Calreticulin's fortuitous association with numerous proteins such as the Ro/SS-A complex (Sontheimer *et al.*, 1994), perforin (Dupuis *et al.*, 1993) and Factor IX (Kuwabara *et al.*, 1993) may suggest that these proteins may require association with calreticulin to reach their final destination.

Calreticulin was found to be associated with PDI in a Zn^{2+} dependent manner (Chapter Six). Both of these proteins have been found to be present in milk lipid droplets during lactation (Ghosal *et al.*, 1994), immunoreactive against sera to LEC patients (Yokoi *et al.*, 1993), and in the acrosome of rat spermatids (Nakamura *et al.*, 1993; Nakamura *et al.*, 1995). Calreticulin and PDI may thus associate with each other and other polypeptides to ensure the proper folding of these proteins within the ER and thus carry then to their final destination. Trafficking studies of how these numerous interacting proteins complexed to PDI/calreticulin are transported within the cell will allow for a proper understanding of how calreticulin may influence the final destination of these proteins and the importance of calreticulin as an ER chaperone.

Has calreticulin found a way to elude the retention/retrieval system of the ER?

Calreticulin has found a way to elude the retention/retrieval system of the ER! Non-ER localization of calreticulin are not fortuitous since calreticulin associations are being discovered or rediscovered by several laboratories. Calreticulin can assume a cytoplasmic localization and even a plasma membrane localization in the presence of UVB radiation on epidermal keritinocytes (Kawashima *et al.*, 1994). How may calreticulin escape the KDEL receptor monitoring system? This still remains a mystery. All the calreticulin species found in the cytosol apparently contain the KDEL retention sequence. Majority of the protein-protein interactions with calreticulin are mediated *via* calreticulin's C-domain (Burns *et al.*, 1993). Hence it is possible that this interaction may mask the functional ER retention signal (KDEL) at the carboxyl terminus of calreticulin that would lead to the secretion of calreticulin along with it's complexed component. It was shown by Sonnichsen *et al.* (1994) that the only way calreticulin was found in a post-ER compartment was through vesicular stomatitis viral infection or deletion of the KDEL and/or high capacity Ca^{2+} binding region of the protein. Overexpressing accounts for only a minor percentage of calreticulin is secreted in COS cells (Sonnichsen *et al.*, 1994). If the interaction point is within the C-domain of calreticulin, then based on the results of Sonnichsen *et al.* (1994) it appears that protein-protein interactions could account for the displacement of this resident ER protein into non-ER compartments.

Is calreticulin an important component of the immune system?

A large number of proteins from diverse areas have been identified with amino acid sequence identity or high homology to calreticulin. These include the Ro/SS-A autoantigen (McCauliffe *et al.*, 1990a), *Schistosoma mansoni* antigen (Hawn *et al.*, 1993), and the onchocercal protein RAL-1 (Unnasch *et al.*, 1988). Antibodies are made against all of these proteins (or the protein, calreticulin) and appear to be related to the predominance of the disease. All of these observations have prompted a re-examination of the potential role(s) this resident ER protein could play in the disease process. The presence of calreticulin in the blood and its anticoagulant activity have further added support that this molecule could be seen by the immune system and hence take part in the disease process. Calreticulin could be an important host cellular factor controlling either the spread or arrest of the invasion process of a foreign body. Evidence for this is observed in the elevated levels of the protein in rubella viral infection (Singh *et al.*, 1994) and in cytomegaloviral infection of human fibroblasts cells (Zhu and Newkirk, 1994). Calreticulin peptide fragments have been observed in association with major histocompatability complex class I molecules (MHC class I). These fragments corresponded to residues 278 - 294 within the sequence of calreticulin (Max *et al.*, 1994) and to the 17mer signal sequence (Henderson *et al.*, 1992). The former peptide was obtained on a HLA-DR4 affinity column after passing Epstein-Barr viral infected B cell lysate. This further supported the hyperactive role of calreticulin during viral infection. MHC class I molecules normally associate with small peptide fragments to form a complex which is then presented to the T-cell receptor. All self antigens will be recognized and not activate the T-cell. During a cellular insult, such as a viral infection, these fragments could get modified so the receptor does not recognize it as self any more. This could then lead to the production of autoantibodies and hence autoimmune manifestations. Evidence to support this hypothesis is observed during rubella viral infection whereby calreticulin gets hyperphosphorylated and thus it interacts with the viral molecule (Singh *et al.*, 1994). Kawashima *et al.* (1994) observed increased cellular and cell surface expression of calreticulin when transformed human epidermal kertinocytes were exposed to UVB radiation. Human sera from patients with SLE, Sjogren's syndrome, rheumatoid arthritis demonstrated a positive antibody response to calreticulin peptides (Routsias *et al.*, 1993). Autoantibody production is observed in diverse areas - affecting the cutaneous layer (in subacute cutaneous lupus erythematosus), the retinal epithelium (in the cause of Onchocerciasis; Braun *et al.*, 1991) or the central nervous system (CNS-SLE; Hanson *et al.*, 1992). The only similarity these various disorders have in common is the immune system and calreticulin. Keyser *et al.* (1992) observed that approximately one-third of lupus patients have antibodies directed against the ribonucleoprotein antigen Sm. This antigen is composed of six protein subunits of which the main antigenic targets are a subunit containing a proline-rich sequence. They proposed that the repetition of certain residues and/or overall positive charge of this epitope may provide a strong signal to override self-tolerance and induce an autoimmune antibody response. Calreticulin's proline-rich P-domain may contribute significantly to the predominance of an autoimmune reaction, and an understanding of how Ca^{2+} is bound within this domain will be required to understand how it may regulate the antigenicity of the protein. Preliminary 1D-NMR looks very promising in providing an impetus for 2D- and 3D-NMR measurements in order to understand the three dimensional structure of the high affinity Ca^{2+} binding site.

Furthermore, immunoreactivity against only a subpopulation of lupus patients would suggest that a considerable proportion of cellular calreticulin molecules, perhaps including secreted forms of this molecule does not have the conformational structure resulting from posttranslational modification or external modification that would allow them to interact with the Ro/SS-A autoantibodies (Sontheimer *et al.* 1994). What is this posttranslational modification or external modification? Does it target the proline-rich area within the P-domain and hence make it antigenic? This would explain the non-reactivity that is observed using the recombinant molecule. Sontheimer and other researchers will have to address these questions in order to understand how this new human autoantigen may be manifested to lead to the disease state in individuals.

In summary, this high affinity Ca^{2+} binding protein identified some 20 years ago, has been found in diverse areas of cell biology and has stimulated a lot of controversy and excitement. Continued research into its structure and regulation in the normal and diseased state will be critical to an understanding of how it can invade such diverse areas of cell biology.

CHAPTER EIGHT

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